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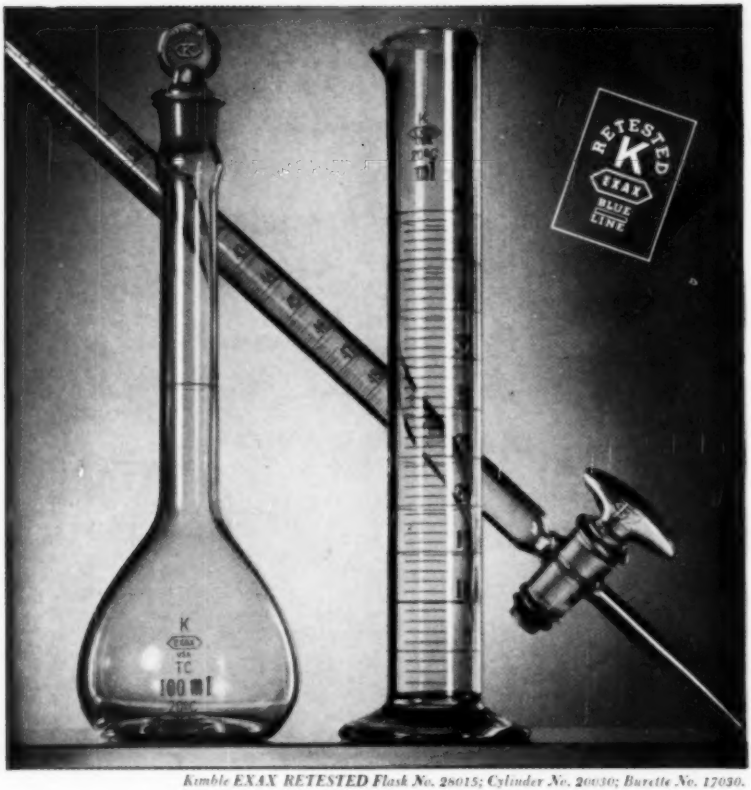

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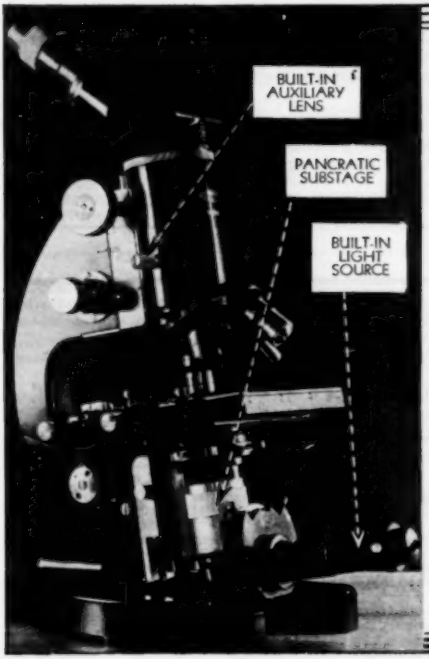

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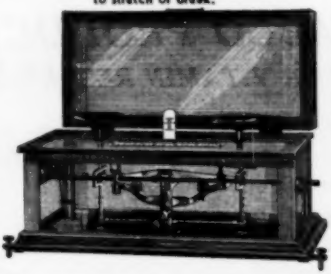

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Acetic Acid as an Irritant in Pyrethrum Extracts*

By ROBERT L. FRANK† and ROBERT L. McGEACHIN†

Highly active dermatitis-producing concentrates have been obtained from pyrethrum flowers by ether extraction and by steam distillation, followed by further fractionation. The most active fractions have been found to be rich in acetic acid, previously unreported in pyrethrum. The suggestion is made that acetic acid contributes to the dermatitis-producing properties of pyrethrum extracts.

IT IS well known that certain individuals working with pyrethrum extracts or flowers have been subject to a dermatitis presumably caused by substances contained in the flowers (1). Martin and Hester (2) obtained active concentrates by ether extraction and by steam distillation of the ground flowers. Feinberg (3) reported that 46 per cent of a group of 225 patients sensitive to ragweed pollen gave positive cutaneous reactions with pyrethrum extracts. Sweitzer (4) has reported a much smaller incidence of sensitive individuals.

The fact that the irritant material is volatile with steam indicates a fairly low molecular weight for the active principle and has encouraged us to investigate further the chemical nature of the dermatitis-producing constituents of pyrethrum. Fractions having irritant properties in sensitive individuals have been obtained in our work from pyrethrum flowers by two methods: ether extraction and steam distillation, both followed by fractional distillation. Various fractions from these experiments were submitted to Dr. A. Rostenberg of the University of Illinois Medical College, Chicago, Ill., and to Dr. S. Mackoff of the University of Minnesota General Hospital, Minneapolis, whose skin tests on sensitive individuals are summarized in Table I.

Of the fractions obtained by ether extraction and distillation, one (Sample No. 7 of Table I) seemed of particular interest because it was not only one of the most potent, but must have consisted entirely of low-boiling material, since it was not condensed in the ice-cooled receiver during distillation of the oleoresin at 2 to 3 mm. pressure. It was collected instead in the dry-ice trap. Investigation showed this material to

* Received August 30, 1948, from the Noyes Chemical Laboratory, University of Illinois, Urbana, Ill.

† We are indebted to the United States Public Health Service for funds which made this work possible. We also wish to thank Dr. C. B. Gnadinger and the McLaughlin-Gormley-King Company of Minneapolis for a generous supply of pyrethrum flowers; Drs. A. Rostenberg, Jr., of the University of Illinois College of Medicine, and S. Mackoff, of the University of Minnesota General Hospital, for finding and patch-testing pyrethrum-sensitive individuals; and Mr. Joseph Dailey for helpful suggestions during the course of the work.

TABLE I.—CUTANEOUS REACTIVITY OF FRACTIONS FROM PYRETHRUM FLOWERS

Sample	Description of Sample	Reaction Patient 1	Reaction Patient 2
Tests by Dr. A. Rostenberg, Jr.[a]			
1	Ground pyrethrum flowers	++++	*
2	Ether-soluble oleoresin from pyrethrum flowers	++++	+++
3	Crystals (m. p. 198°) from ether extract before distillation (probably chrysanthin)	*	−
4	Ground pyrethrum flowers after ether extraction	?	−
5	Alcoholic extract of residue after distillation of oleoresin obtained by ether extraction	−	−
6	Distillate from ether extract (material volatile at 2–3 mm. pressure with bath temperatures up to 250°); n^{25}_{D} 1.4208	+++	+++
7	Low-boiling distillate from distillation of oleoresin obtained by ether extraction (collected in dry-ice trap); n^{25}_{D} 1.3890	++++ (large blister)	++++
8	Oil from ether extract of steam distillate of pyrethrum flowers	++++	++
9	Same as no. 8	++++	*
10	Oil from ether extract of steam distillate of pyrethrum flowers, in which the steam distillate was made alkaline with sodium bicarbonate before extraction	++++ (large blister)	++++
Tests by Dr. S. Mackoff[b]			
11	Oil from ether extract of steam distillate of pyrethrum flowers, the distillation carried out with return of water to still	−	....
12	Distillate from ether extract of steam volatile oil, b. p. 80–84°/5 mm.; n^{25}_{D} 1.4855	−	....
13	1% aqueous acetic acid	−	....

[a] Tests carried out by Dr. A. Rostenberg, Jr., of the University of Illinois College of Medicine.
[b] Tests carried out by Dr. S. Mackoff of the University of Minnesota General Hospital on six patients who gave positive reactions with pyrethrum flowers.

contain approximately 30 per cent acetic acid, characterized by boiling point, refractive index, Duclaux constants, and the formation of a crystalline *p*-bromophenacyl ester.

The occurrence of acetic acid in pyrethrum flowers is of interest because it has apparently not been previously reported.[1] It is of further interest because a 30 per cent solution of acetic acid is irritating to the skin of most persons.[2] Thus arises the natural suggestion that some of the irritation of individuals to pyrethrum oleoresins is due to acetic acid, concentrated during the extraction process.

Examination of Table I brings out the fact that the active fractions are just those in which acetic acid would be concentrated during their preparation. Even Sample 10, an ether extract from the steam-volatile oil made alkaline before extraction, must contain acetic acid, since it can be readily demonstrated that acetic is a weak enough acid to be extracted by ether from an aqueous alkaline medium.

The same statement, i.e., none of the irritant fractions described so far can be said with certainty to be devoid of acetic acid, appears to hold true for the fractions tested by Martin and Hester (2).

It is not to be inferred from these findings that acetic acid is the main allergenic principle in pyrethrum flowers. Such a conclusion is denied by the fact that ground pyrethrum flowers alone, unconcentrated in acetic acid, give a strong reaction in some individuals.[3] Further, the extreme effects on J. T. Martin of the extracts studied by Martin and Hester can hardly be ascribed to the main ingredient in household vinegar. On the other hand, the nonspecific skin irritation caused by acetic acid is similar in appearance to that evoked by the specific allergen of pyrethrum flowers, and we believe that a number of the extracts tested by Martin and Hester and by others probably contained enough acetic acid to act as a primary irritant. This may account for the disturbing observation of Martin and Hester in their excellent report that all their fractions, obtained by various means, gave positive reactions, though of varying intensities, and that all these fractions had, to different degrees, the sour odor of pyrethrum flowers.

The relative amounts of acetic acid in various extracts may also explain the divergence in the reported incidence of pyrethrum dermatitis

[1] The odor of dried pyrethrum flowers is probably partly due to the presence of acetic acid. The odor of very dilute solutions of the latter markedly resembles the faintly sour smell of pyrethrum.

[2] Experiments in this Laboratory have indicated that most normal individuals produce a red welt after an exposure of one hour to a gauze patch moistened with 10% aqueous acetic acid.

[3] A 1% aqueous acetic acid solution gave a negative reaction on a patient sensitive to the dried flowers (Table I, Sample 13).

(3, 4), mentioned in the introduction to this report. Studies with the flowers themselves, however, are not likely to be influenced by acetic acid, since before extraction the latter is present in amounts of less than one per cent.

Thus far the complete removal of acetic acid from our active concentrates has resulted in total loss of cutaneous activity.

EXPERIMENTAL

Ether Extraction of Pyrethrum Flowers.—Twenty pounds of powdered Kenya pyrethrum flowers (Table I, Sample 1) were extracted (in one-pound lots in a Soxhlet extractor) with ethyl ether (2 L. of ether per pound of flowers) to give an ether solution having a dark green color. The ether was removed slowly by distillation at atmospheric pressure. When approximately four-fifths of the ether had been removed, a small quantity of colorless rhomboid crystals separated. The ether solution was decanted from the crystals, which were then removed, washed well with cold ether, and air dried (Table I, Sample 3). From their melting point, 198° (201° corr.), they were judged to be chrysanthin (5).

The remainder of the ether was then removed by distillation at atmospheric pressure and further by evacuation to 20 mm. pressure. The residue was a dark green semisolid oleoresin weighing 1400 Gm. (Table I, Sample 2).

This residue was then subjected to distillation without fractionation through a short distilling head at 3–4 mm., with bath temperatures increasing to 250°. A fraction of 25 ml. was collected in a receiver cooled by an ice-bath (Table I, Sample 6); another 25-ml. fraction was obtained in a dry-ice trap connected to the receiver (Table I, Sample 7). The former fraction contained some water, which separated as a separate phase. The latter fraction was clear and colorless, but turned reddish brown on standing. It had a few crystals floating on its surface; these were removed, recrystallized three times from 50% aqueous ethanol and dried *in vacuo*, m. p. 48–50°.

Anal. Found: C, 83.10; H, 14.01. These were not further characterized.

The material in the dry-ice trap was found to be approximately 30% acetic acid, as described below.

Characterization of Acetic Acid.—A 1.0-ml. portion (sp. gr. 0.90; n_D^{20} 1.3745) of the liquid obtained above in the dry-ice trap was diluted with 15 ml. of water and titrated with 0.12 *N* sodium hydroxide, using phenolphthalein as indicator. It required 37.8 ml. to neutralize the acid, indicating, on the assumption that all the acid present was acetic, an acetic acid content of 29.9% in the fraction.

Three milliliters of the same material was diluted with 100 ml. of water. Ten milliliters required 9.9 ml. of 0.1 *N* potassium hydroxide for neutralization. On distillation successive 10-ml. portions required 6.8, 7.4, and 8.1 ml. of alkali. The Duclaux numbers for acetic acid are 6.8, 7.1, and 7.4.

The solutions of potassium acetate formed in the determination of the Duclaux numbers were combined, evaporated to dryness, and the potassium acetate fused. This salt was used to prepare a *p*-bromophenacyl ester, m. p. 84° (lit. (6), 85°).

Steam Distillation of Pyrethrum Flowers.—(1) One hundred and fifty grams of powdered pyrethrum flowers were steam distilled for approximately thirty hours (until no more oil could be detected in the condensate). The distillate was then saturated with sodium chloride and extracted with ethyl ether. The extracts were dried over anhydrous sodium sulfate and the ether evaporated to give 0.5 ml. of orange-yellow oil having a sour odor (Table I, Samples 8 and 9).

(2) Eight lots of 2000 Gm. each of powdered pyrethrum flowers were steam distilled, each lot being steam distilled about thirty hours (until no more oil could be detected in the liquid distilling). The apparatus used was such that the water layer from the distillate was continuously returned to the distilling flask. The oil was lighter than water so that it remained on the surface. The distillate from each lot was extracted with three 100-ml. portions of ether, the ether extracts from all the runs being combined and dried over anhydrous magnesium sulfate. The ether was then removed by distillation at atmospheric pressure and a bath temperature of 45–50°. When most of the ether had been removed, the temperature of the bath was raised to 80°, whereupon a volatile liquid distilled. This had an odor resembling that of acetaldehyde and gave a positive reaction with fuchsin-aldehyde reagent. The quantity was too small for further investigation. The liquid remaining was viscous; light orange in color; had a sour odor; and was distinctly acid to litmus (Table I, Sample 11). It is to be noted that this second means of steam distillation in which the water is returned to the flask fails to concentrate enough acetic acid to give a positive skin test. The yield was 16.7 Gm., or 0.1%, of the dry weight of the flowers.

This liquid was fractionally distilled through a small Podbielniak column to give 1.5 Gm. of crude acetic acid, n_D^{20}, 1.3841 (all the material distilling below 47° (10 mm.) and nine approximately equal fractions of b. p. 47–95° (5 mm.) having n_D^{20} ranging from 1.4856 to 1.4862. A middle fraction (Table I, Sample 12), although inactive as an irritant, was further characterized as follows: it was a viscous yellow oil, insoluble in water but soluble in ethanol and ethyl ether; sp. gr.$_{25}^{25}$, 0.948. It gave an acid reaction with litmus and had a neutral equivalent of 343. It decolorized bromine in carbon tetrachloride solution with no evolution of hydrogen bromide, and also decolorized alkaline potassium permanganate. It contained no nitrogen (Dumas), sulfur (sodium fusion), nor halogen (Beilstein test).

Anal. Found: C, 75.81, 75.74; H, 10.00, 9.91; C-Methyl: 12.0, 11.7%.

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Observations on the Mode of Action of Metrazol in Convulsive Doses*

By MAYNARD MURRAY, C. ROWELL HOFFMANN, and FRED R. SCROGGINS

AN INCREASE in the guanidine content of the blood of epileptics has been reported by Ellis (1). In a recent communication (2), it was shown that there was not only a slight increase in the basal level of guanidine-like substances in the blood of patients with epilepsy, but that there was a synchronous rise in the quantity of these substances in the blood with the convulsion (Fig. 1). We were interested to determine whether this rise in blood guanidine levels occurred in artificially induced fits as well as in spontaneous ones, i.e., whether the guanidine rise

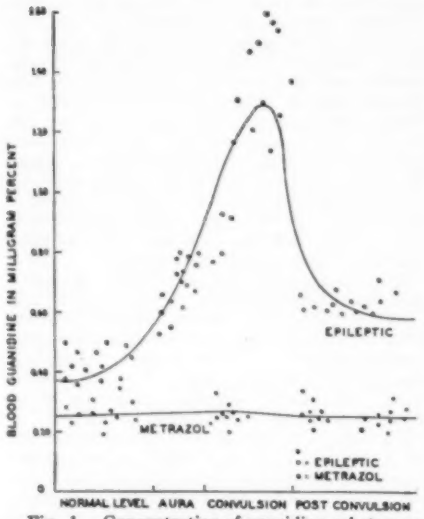

Fig. 1.—Concentration of guanidine substances in the blood in metrazol and epileptic convulsions.

was a result of the convulsion *per se*, or whether there was a difference in this biochemical reaction in the two types of fits.

The Effect of Metrazol Shock on Blood Guanidine[1] Levels

A group of ten patients at Longview State Hospital who were receiving metrazol shock therapy was studied. The same method for the determination of guanidine-like substances in the blood was employed as that used in the previous study on idiopathic epileptics. The normal blood guanidine level, and the levels at periods of one, fifteen, and sixty minutes after injection of metrazol in convulsive doses were ascertained in each case. The results of these determinations are given in Table I.

A comparison of the alteration in blood guanidine levels in spontaneous and in metrazol fits is represented graphically in Fig. 1. It will be seen that in this series of metrazol convulsions on ten patients all the values fill within the normal range, as reported by Andes and Myers (3), and that there was no rise in the blood guanidine levels in these fits comparable to that seen in spontaneous convulsions in idiopathic epileptics.

The Effect of Metrazol Shock on the Gonads

Investigators are generally agreed that many patients with involutional melancholia are improved markedly by metrazol shock therapy. This disorder is commonly considered to be due to a gonadal hormone insufficiency. Since it has been demonstrated by Marshall & Verney (4) that ovulation of

TABLE I.—GUANIDINE VALUE IN MG. % IN METRAZOL CONVULSIONS

Patient's No.	Normal Level	One Minute After Metrazol	Fifteen Minutes After Metrazol	Sixty Minutes After Metrazol
16	0.23	0.23	0.14	0.21
17	0.27	0.25	0.26	0.26
18	0.24	0.29	0.31	0.28
19	0.31	0.33	0.34	0.32
20	0.23	0.24	0.24	0.24
21	0.26	0.25	0.25	0.25
22	0.25	0.25	0.24	0.23
23	0.26	0.26	0.27	0.25
24	0.28	0.27	0.27	0.27
25	0.19	0.20	0.21	0.20

central origin through the anterior pituitary may be induced in the rabbit by electrical stimulation of either the cerebrum or the lumbosacral cord, and by convulsions produced by the intravenous injections of picrotoxin (5), the following question occurred: Does metrazol shock exert its beneficial therapeutic effect in these cases by acting directly upon the gonads, or upon gonadotropic secretions of the anterior pituitary gland?

An attempt to answer this question was made. Because of their peculiarly prompt response to estrogens and to gonadotropic anterior pituitary fractions, female rabbits of twelve to fourteen weeks of age were chosen as test animals, since any anterior pituitary gonadotropic principle would in all likelihood cause ovulation (6), while follicular activity would cause endometrial hyperplasia (7).

* Received Nov. 13, 1948, from the Physiology Laboratory, University of Cincinnati. This work was done in 1940 but interrupted by the authors' service with the Armed Forces.

[1] Throughout this article the term guanidine is used in lieu of guanidine-like substances.

Twelve female rabbits weighing between 2400 Gm., and 2800 Gm., were given intravenous injections of 0.20 Gm. of metrazol. Immediately the animals were thrown into violent convulsions, lasting between ten and fifteen minutes. With the exception of one animal, which broke several teeth as a result of spasm of the muscles of mastication, no injuries or fractures were observed. Following this, laparotomies were performed on the animals, in groups of three at twenty-four, forty-eight, seventy-two, and ninety-six hours, respectively. The ovaries, tubes, and uteri were removed. In no case did examination show changes in the ovaries such as congestion, enlargement, developing follicles or corpora hemorrhagica or leutes. A study of sections of the uterine mucosa showed no evidence of endometrial hypertrophy.

Another group of twelve female rabbits of the same age and weight range were given metrazol convulsions on successive days for seven days, and then forty-eight hours after the last convulsion, laparotomies were performed. In no case were ovarian changes noted, nor were any changes noted in the uterine mucosa when sections of this organ were studied.

CONCLUSION

Metrazol fits are not attended by a rise in the level of blood guanidine as are spontaneous convulsions in idiopathic epileptics, indicating a different chemical reaction in the two types.

Metrazol convulsions cause no changes in the ovaries or the endometrium of female rabbits indicating that this therapy, although effective in involutional melancholia, does not act in any demonstrable fashion on the anterior pituitary gland or the gonads.

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Qualitative and Quantitative Determination of Peroxides in Petrolatum*

By MILTON J. GOLDEN

A simple and accurate qualitative and quantitative Peroxide Test method is proposed for the determination of peroxides in petrolatums. The amount of peroxides in a petrolatum can be determined chemically by the oxidation of the ferrous ion to the ferric ion by the peroxides, utilizing the corresponding development of a pink or red color. Samples of petrolatum have been tested accordingly, and a table is given which compares the quantitative Peroxide Values of the petrolatums with their respective Peroxide Tests. A Peroxide Test of at least two hours is suggested as one of the minimum standards for petrolatums used in the manufacture of pharmaceuticals and cosmetics.

PETROLATUM, a substance closely related to White Mineral Oil, has become, in recent years, an important constituent of pharmaceutical and cosmetic preparations. In contrast to mineral oil, which is a liquid under normal conditions, petrolatum is semisolid at ordinary temperatures, consisting of a mixture of solid and liquid hydrocarbons. It is amorphous and microcrystalline and may be regarded as a colloidal system in which two phases are present, an external phase of solid wax and an oily internal phase.

In the selection of the proper type of petrolatum for a specific purpose, several important properties must be considered, namely fiber characteristic, melting point, and consistency. It seems that for most pharmaceutical and cosmetic preparations, a medium fiber is generally used. A short fiber petrolatum, characteristic of synthetic petrolatums and those refined by chemical treatment, is undesirable since it tends to produce a thin body and lacks the smooth consistency usually found with medium fiber. A long fiber petrolatum, on the other hand, produces a sticky and stringy finished product. In addition to the fiber characteristic, the melting point demands consideration and should be sufficiently high so that the finished preparation will not liquefy at slightly elevated temperatures. Furthermore, correct and uniform consistency is by far one of the most important factors in determining the suitability of a petrolatum.

The Pharmacopœia of the United States has recognized the importance of the purity of petrolatum and has, therefore, included tests for ash content, organic and mineral acids, alkali

* Received Nov. 8, 1948, from the Research Laboratories, McKesson & Robbins, Inc., Bridgeport, Conn.

and color, in addition to the properties already mentioned. It does not, however, include any specific test for determining stability.

As in the case with mineral oil (1), petrolatum is also known to develop peroxides upon heating and prolonged standing, which are objectionable and undesirable. It is important that petrolatums which are to be used in the preparation of pharmaceuticals and cosmetics should resist deterioration and be stable for a reasonable length of time in respect to odor, color, and development of peroxides. Moreover, this factor of stability is of major importance and particular interest to those pharmaceutical and cosmetic manufacturers who subject petrolatum to high temperature for various periods of time.

The so-called Peroxide Number method has been suggested for determining the amount of peroxides in petrolatums (2). However, various sources of error exist in this method. There may occur the reabsorption of the liberated iodine by the petrolatum and the oxidation of the reagents by air. Furthermore, the peroxides in the petrolatum do not seem to react readily with the potassium iodide. Consequently, the values obtained are not reproducible and reliable.

The purpose of this paper is to introduce a simple and accurate method for the qualitative and quantitative determination of peroxides in petrolatum.

EXPERIMENTAL

It has been shown in previous reports that the peroxide content of mineral oils can be determined chemically by the oxidation of the ferrous ion to the ferric ion by the peroxides formed in heat-treated oils (1, 3). This oxidation can be measured colorimetrically.

In the Peroxide Test for Mineral Oils (1), a colorless test solution of ferrous sulfate, ammonium thiocyanate, sulfuric acid, reduced iron and iron wire is added to samples of mineral oil heated in a 300° F. oil bath for various time intervals and the developing color observed. The color varies from colorless to dark red, depending upon the amount of peroxides formed during the breakdown of the oil. The first sample exhibiting a pink coloration after shaking with the test solution is regarded as the Peroxide Test value expressed in minutes.

These facts and reactions have been used in the development of the following qualitative and quantitative procedure for detecting and estimating the amount of peroxides formed in petrolatums.

The consistency and structure of petrolatum necessitated the use of a solvent which had to be miscible with the petrolatum and not with the test solution. Several organic solvents have been tried and found to be unsuitable due to their miscibility with the acetone contained in the test solution. Only mineral oil possesses the required properties. The mineral oil was of high purity and had a Peroxide Test of at least thirty-five minutes.

In the Peroxide Test method, samples of petrolatum are heated at 250° F., the temperature equivalent to 15 pounds of steam pressure, for various time intervals from one-half hour to several hours and then mixed with mineral oil, heated to the same temperature in the ratio of 2:3. The petrolatum-mineral oil mixture is then cooled to 170° F. Subsequently, the test solution, consisting of ferrous sulfate, ammonium thiocyanate, sulfuric acid, iron and iron wire, is added, and the change in color of the lower test solution layer from pink to darker pink or red, depending upon the amount of peroxides formed, is observed and measured. Since there are always some peroxides present in petrolatum, the formation of a pink color in the test solution layer with unheated samples is to be expected.

Apparatus.—One-liter round-bottom flask and reflux condenser; test tubes $^3/_4$ x 6 inches; constant temperature oil bath regulated at 250° F. ± 1° F.; glass-stoppered reaction flasks with greaseless stopcock, according to Connor and Staub's vitamin B_1 fluorometric method; centrifuge; Klett-Summerson photoelectric colorimeter with filter No. 56, having a mean transmission of 560 mμ, and special test-tube cells.

Materials.—Ferrous Sulfate C. P.; Ammonium Thiocyanate, C. P.; Iron by Hydrogen, Merck (reduced iron); Iron Wire No. 30, for standardization; Acetone U. S. P.; 3% Hydrogen Peroxide Solution.

Preparation of Reagent.—In a round-bottom, wide-mouthed flask, 2 Gm. of ferrous sulfate is dissolved in 100 cc. of distilled water containing 2 cc. concentrated sulfuric acid. Two hundred cubic centimeters of acetone and 100 cc. of a 2% aqueous solution of ammonium thiocyanate are then added to the above solution. Iron powder 0.2 Gm. and 6 inches of No. 30 iron wire are finally added to reduce any trace of ferric ion present. The resulting mixture is refluxed on a steam bath until the red color of the solution has disappeared. This reduction process takes about two hours, it being necessary to continue refluxing to insure lack of color.

Fresh solution is prepared on the day of the test. It is possible, however, to preserve it in a colorless state by careful storage in a carbon dioxide atmosphere.

Since it is necessary to avoid any contact with air, a dispensing method has been devised. A siphon is passed into the upper end of the reflux condenser through a two-hole cork stopper. The second hole is fitted with a glass stopcock, which serves as a vent. Carbon dioxide is admitted through the vent to inaugurate the flow of the solution, the pressure forcing the liquid into the siphon, at the end of which is attached a glass stopcock for the withdrawal of the solution.

Qualitative Procedure.—Petrolatums to be tested are immersed in a constant temperature oil bath at 250° F. ± 1° F. Two-cubic centimeter samples of this heated petrolatum are removed after one-half hour and at hourly intervals thereafter and then mixed with 3 cc. of mineral oil heated to the same temperature in individual test tubes. The petrolatum-mineral oil mixture is then cooled to 170° F. The air in the test tubes is replaced with carbon dioxide and 5 cc. of the test solution is added. The air in the tube is again replaced with carbon dioxide. The tube is tightly stoppered with a new cork stopper and shaken by hand for fifteen seconds. The

formation of additional peroxides in the petrolatum is detected by the development of a darker pink to red coloration of the bottom layer in the test tubes. The first tube exhibiting a change in color from pink to darker pink or red after shaking with the test solution is regarded as the Peroxide Test Value expressed in hours.

Quantitative Procedure.—The colorimetric Peroxide Test Method (3), recently proposed for determining the amount of peroxides formed in mineral oil, has been modified and adopted for petrolatums. Samples of petrolatums to be tested are immersed in a constant temperature oil bath at 250° F. ± 1° F., the temperature equivalent to 15 pounds of steam pressure, and then mixed with mineral oil heated to the same temperature in the ratio of 2:3. A 10-cc. sample of the heated petrolatum-mineral oil mixture, cooled to 170° F., is pipetted into a glass-stoppered reaction flask which has been marked to indicate 10-cc. and 20-cc. filling. The air in the flask is replaced with carbon dioxide, and 10 cc. of the colorless test solution is added. The flask is then tightly stoppered with the glass stopper, shaken by hand for one minute, and centrifuged for three minutes. The lower test solution layer which will then vary in color from pink to dark red is transferred by means of the greaseless stopcock from the reaction flask to a cork-stoppered special Klett test-tube cell, in which the air has also been replaced with carbon dioxide. As a control, 10 cc. of the mineral oil, heated to 250° F. and then cooled to 170° F., is treated similarly and run parallel with the heated sample. The test-tube cells, containing the sample and control, are then placed immediately in the Klett colorimeter, and the amount of pink or red color formed is measured using the Klett filter No. 56. The difference in scale readings between the sample and control gives the reading for the oxidation in the test solution by the peroxides in the melted petrolatum. With certain types of petrolatums, the turbidity of both control and sample must be considered in order to obtain reproducible values. The turbidity of both solutions is measured with the Klett filter No. 66, and the values obtained are then subtracted from the original readings.

Prior to the above-described procedure, the Klett photoelectric colorimeter is adjusted until a zero reading is obtained with the colorless test solution and then standardized with various concentrations of hydrogen peroxide by measuring the amount of color corresponding to various quantities of peroxide. A freshly prepared 3% hydrogen peroxide solution is diluted 1–100, and its peroxide content is determined according to the U. S. P. assay method with 0.01 *N* potassium permanganate solution. Very small volumes of this diluted peroxide solution, having a concentration range of 1×10^{-4} to 1×10^{-4}, are added to 5-cc. portions of the colorless test solution in cork-stoppered Klett test-tube cells, and the amount of pink or red color formed is measured in the colorimeter.

Hydrogen peroxide concentrations are plotted against scale reading, and a standardization curve is obtained with increasing concentrations of peroxides. The resulting graph facilitates the determination of the amount of peroxides in any sample of petrolatum by evaluating the concentration corresponding to the difference in scale readings between control and sample.

DISCUSSION

These Peroxide Test procedures make possible not only the evaluation of the peroxides in petrolatums, but also the comparison of those petrolatums which, with the known methods of testing, would appear to have similar stability and quality.

Various types of petrolatum were tested accordingly. Table I summarizes the comparison of the Peroxide Values of representative samples of three types of petrolatum heated at 250° F. for various time intervals with their respective Peroxide Tests. The Peroxide Value is a quantitative expression for the amount of hydrogen peroxide formed in a kilo of petrolatum heated to 250° F. The Peroxide Test, on the other hand, is a qualitative test in which hours of heating and detection of the degree of peroxides formed provide a means of establishing the stability of petrolatums.

The Peroxide Value reported for each sample represents the average of at least three separate determinations, which never varied more than ±3%.

The accuracy and reproducibility of the above-described colorimetric method have been studied by means of dilute solutions of hydrogen peroxide of known concentration and by means of pure preparations of succinyl peroxide. For example, the concentration of hydrogen peroxide solution as given by the standard potassium permanganate method was 0.0036 mole per L., while the colorimetric procedure gave 0.0037 mole per L. The succinyl peroxide was prepared by the method of Clover and Houghton (4) and was recrystallized twice from acetone. The calculated weight per cent of active oxygen in succinyl peroxide is 6.83%; obtained by the colorimetric procedure 6.92%.

The results obtained confirm and substantiate what has already been reported, namely, that heat will accelerate the breakdown of petrolatum with the formation of additional peroxides (2).

These suggested Peroxide Test methods offer a means of determining a new constant or property for petrolatums to be used in pharmaceutical and cosmetic preparations. In the pharmaceutical use of petrolatum, it is of the utmost importance that careful consideration be given to the factor of stability. The values obtained by the above-described procedures offer themselves as a yardstick in this respect.

TABLE I.—COMPARISON OF PEROXIDE VALUES WITH PEROXIDE TESTS OF VARIOUS TYPES OF U. S. P. PETROLATUMS HEATED AT 250° F. FOR VARIOUS TIME INTERVALS

Type	Peroxide Values, Gm. of Hydrogen Peroxide per Kilo of Heated Petrolatum				Peroxide Test, Hr.
	0 Hr.	2 Hr.	3 Hr.	4 Hr.	
Amber	2.7×10^{-3}	2.8×10^{-3}	4.5×10^{-3}	5.5×10^{-3}	3
Snow White	1.7×10^{-3}	2.4×10^{-3}	5.7×10^{-3}	6.2×10^{-3}	2
Lily White	1.6×10^{-3}	1.7×10^{-3}	1.4×10^{-2}	1.7×10^{-2}	3

Petrolatums used in the preparation of pharmaceuticals and cosmetics should resist deterioration and be stable for a reasonable length of time in respect to odor, color, and the development of peroxides.

SUMMARY

A qualitative and quantitative Peroxide Test method is proposed for the determination of peroxides formed in petrolatums. These methods are simple and accurate.

A Peroxide Test of at least two hours is herewith suggested as one of the minimum standards for petrolatums used in the manufacture of pharmaceuticals and cosmetics.

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The Microbiological Determination of Choline*

By F. J. BANDELIN

The microbiological method for the determination of choline utilizing the growth response of the cholineless mutant of the mold, *Neurospora crassa*, is more sensitive and more specific than the chemical methods generally employed. It is not, however, as sensitive as microbiological methods for many other biologically essential substances such as the vitamins. Methionine, which interferes only slightly in the determination, may be removed by adsorption. The reagents, method, and preparation of samples are given, along with data obtained from determinations on various biological products generally used in the pharmaceutical industry. Reproducibility of results and recovery of added choline indicate that the method is reliable and satisfactory for these materials.

CHOLINE HAS been known as a chemical entity since Strecker first isolated it from hog bile in 1849 (1). Not until recently, however, has its important function as a lipotropic agent in nutritional physiology become known.

It is ubiquitous in nature, occurring in living tissues, both animal and vegetable, largely in the form of phospholipids; to a lesser extent as free choline; and in minute quantities as acetylcholine. In view of its new-found importance and wide distribution, it is natural that methods of isolation and determination be investigated in order to evaluate the natural sources of choline.

Chemical methods investigated to date are neither as highly sensitive nor are they as specific as for other biologically essential substances such as the vitamins. The microbiological method based upon the use of a cholineless mutant of the mold *Neurospora crassa* developed by Horowitz and Beadle (2) is considerably more sensitive and more specific than any chemical method yet reported. The mutated strain is produced by ultraviolet irradiation of the wild *Neurospora crassa* which when so treated loses its ability to synthesize choline and consequently requires exogenous choline for growth. The mutant therefore is designated as cholineless, strain No. 34486. Growth of the cholineless *Neurospora* is stimulated not only by choline but also by the following choline analogs: acetylcholine, dimethylaminoethanol, monomethylaminoethanol, phosphorylcholine, arsenocholine, and by *dl*-methionine. Fortunately, with the possible exception of *dl*-methionine, the concentrations of these compounds in biological material are insignificant as compared with choline and hence do not interfere. Even methionine is only 0.002 as active as choline (2) on a molar basis and may, in most instances, be ignored in choline assays since the ratio of choline to methionine in most substances is such that any growth stimulation of the *Neurospora* due to methionine is within the experimental error inherent in the method. Sarcosine, betaine, creatine, ethanolamine, dimethylamine, trimethylamine, tetramethylammonium hydroxide and chloride do not interfere. Lecithin may serve as a source of choline for the *Neurospora* but the growth response is slow, usually not more than 40 per cent of the total choline being accounted for in three days' incubation. This is no doubt due to the poor solubility and slow hydrolysis of lecithin.

* Received Jan. 17, 1949, from the Research Laboratories, Flint, Eaton and Company, Decatur, Ill.

Where the concentration of methionine and certain nonbasic substances is such that it interferes in the determination, they may be removed through base exchange by passing the solution through a Zeolite column. The eluate is used for the determination.

PROCEDURE

By the following method it is possible to determine from 2 to 20 γ of choline in biological materials with a considerable degree of accuracy and reproducibility. Results of determinations made upon various pharmaceutical products and certain raw materials used in pharmaceutical products are given.

Reagents:

3% Sulfuric Acid
Saturated Solution Barium Hydroxide
1 *N* SodiumHydroxide
0.3% Sodium Chloride Solution
5.0% Sodium Chloride Solution
Standard Solution No. 1: Contains 1.0 γ of choline chloride per ml.
Standard Solution No. 2: Contains 10 γ of choline chloride per ml.
Basal Medium (Gm. per 500 cc.)

Ammonium Tartrate	5.0 Gm.
Ammonium Nitrate	1.0 Gm.
Potassium Phosphate (monobasic)	1.0 Gm.
Magnesium Sulfate (heptahydrate)	0.5 Gm.
Sodium Chloride	0.1 Gm.
Calcium Chloride	0.1 Gm.
Sucrose	20.0 Gm.
Biotin	5 γ
Asparagine	1.0 Gm.
Glutamic Acid	1.0 Gm.
Trace Salts Solution[1]	1 cc.

Adjust the *p*H to 5.0 and dilute to 500 cc.

Stock cultures of the cholineless *Neurospora crassa* are maintained on agar slants prepared from the basal medium by the addition of:

Agar	1.5%
Difco Yeast Extract	0.2%
Malt Extract	0.2%
Choline Chloride	1 γ per ml.

The slants are prepared in cotton plugged bacteriological tubes using 6 ml. of the above agar mixture and slanting to cool after autoclaving at 15 pounds pressure for ten minutes.

PREPARATION OF THE SAMPLE

A sample of from 50 to 500 mg. is weighed into a 50-ml. Erlenmeyer flask. Ten milliliters of 3% sulfuric acid is added; the flask is plugged with cotton and autoclaved at 15 lb. pressure for two hours.[2]

[1] Trace Salts Solution:

	Mg. per L.
Boron (as sodium borate)	0.01
Molybdenum (as ammonium molybdate)	0.02
Iron (as ferrous sulfate)	0.2
Copper (as copper sulfate)	0.1
Manganese (as manganese sulfate)	0.02
Zinc (as zinc sulfate)	2.0

[2] Refluxing with 5% sulfuric acid for five hours also apparently liberates all bound choline.

After cooling, the material is quantitatively transferred, with the aid of 5 ml. of water, to a 50-ml. centrifuge tube and neutralized to Congo Red by the dropwise addition of saturated barium hydroxide solution. The barium sulfate formed by this step, together with any undissolved residue, is centrifuged down and the supernatant is filtered through a No. 50 Whatman paper. Three milliliters of distilled water is added to the residue in the tube and stirred with a glass rod. The tube is immersed in a boiling water bath for ten minutes with occasional stirring. The contents are cooled by holding the tube under the tap and then they are centrifuged. This washing is added through the filter to the previous supernatant. The clear filtrate is neutralized to litmus with 1 *N* sodium hydroxide and made up to volume with distilled water in a volumetric flask. This solution or subsequent dilutions should be prepared, based on the estimated choline content, so that the final dilution contains from 2.0 to 5.0 γ per ml. calculated as choline chloride.

If base exchange is necessary for the removal of interfering substances, 5 ml. of the neutralized solution is passed slowly through a Decalso column of the type generally used in vitamin separations. If the solution contains less than 1 γ of choline per ml., 10 ml. is passed through the column. It is then washed with two 5-ml. portions of 0.3% sodium chloride solution. A graduated test tube or cylinder is placed under the column and the choline is eluted with 10 ml. of hot 5% sodium chloride solution. The eluate, when cool, is adjusted to exactly 10 ml. with distilled water. Two or more samples should be run simultaneously at the same level.

ASSAY PROCEDURE

Ten-milliliter portions of basal medium are pipetted into 125-ml. flasks. The final volume, in each case, is adjusted to 20 ml. by the addition of test solution and water. For each series of determinations a standard series is set up containing 0.5, 1, 2, 3, 4, 5, 7, 10, 15, 20, 25, 30, 40, 50, and 100 γ of choline chloride per flask. This is accomplished by using 0.5, 1, 2, 3, 4, 5, 7, and 10 ml. of standard solution No. 1 containing 1 γ of choline chloride per ml. and then using 1.5, 2.0, 2.5, 3.0, 4, 5, and 10 ml. of standard solution No. 2 containing 10 γ of choline chloride per milliliter. Test solutions may be run at several levels if desired. A convenient dilution of the unknown is one containing, in each ml., from 2 to 5 γ of choline calculated as the chloride.

After addition of the choline solutions to the flasks, the volume is adjusted to 20 ml. with distilled water. The flasks are then plugged with cotton and autoclaved at 15 lb. pressure for ten minutes. After cooling to room temperature, each flask is inoculated with one drop of a suspension of the *Neurospora conidia* in sterile water. This suspension is prepared by scraping some of the mold from the slant with a sterile wire loop and transferring it with sterile technique to a test tube containing from 5 to 10 ml. of sterile water. The tube is well shaken to disperse the *conidia* after which the suspension is withdrawn with a sterile pipette or dropper and one drop used to inoculate each of the flasks of media and test solution.

The flasks are incubated at 30° for seventy-two hours and the resulting orange-colored mold pads

harvested by filtering each through a tared, sintered, glass crucible of medium porosity. The pads in the crucibles are washed with 10 ml. of distilled water and dried at 80°. After cooling in a desiccator the crucibles containing the mold pads are weighed and the weight of the mold pads determined. Choline values are calculated as the chloride in the usual way from a plot of the standard series.

A typical curve obtained by plotting a standard series is given in Fig. 1.

The detailed results obtained from determinations on a number of pharmaceutical biological products run at several levels, as well as recovery of added choline chloride, are given in Table I.

Results obtained on various dilutions of the different materials, both with and without base exchange, are given in Table II.

DISCUSSION

The growth response of the cholineless *Neurospora* is a linear function of the concentration over a rather narrow range. The optimum working range seems to be from 2 to 20 γ. The test solution should be prepared to have a concentration of from 2 to 5 γ of choline, as the chloride, per ml. where this is possible. Because of the difference in response of the *Neurospora* from time to time, standard

TABLE I.—RESULTS OF DETERMINATIONS ON A NUMBER OF PHARMACEUTICAL BIOLOGICAL PRODUCTS AND RECOVERY OF CHOLINE CHLORIDE

Material	Filtrate Used, Ml.	Choline Chloride Added, γ	Dry Weight of Mold, Mg.	Choline Found (as Chloride) γ per Flask	Choline Found (as Chloride) Mg. per Gm. of Sample	Choline Chloride Recovered γ
Liver Extract Injectable, 2 U. S. P. Units	1.0	0	19, 20	2.5	12.5	...
	2.0	0	43, 41.5	5.2	13.0	...
	3.0	0	50.5, 49	7.7	12.8	...
	1.0	5.0	50, 50	7.8	...	5.3
	2.0	5.0	55, 56	10.0	...	4.8
Liver B, Vitamins Injectable	1.0	0	35, 37	4.2	14.5	...
	2.0	0	53, 51	8.8	15.4	...
	3.0	0	60, 62.5	13.1	15.3	...
	1.0	5.0	54, 54	9.0	...	4.8
	2.0	5.0	62, 63.5	14.0	...	5.2
Liver Extract Injectable, 5 U. S. P. Units	1.0	0	15, 16	2.8	0.24	...
	2.0	0	31, 34	4.8	0.24	...
	3.0	0	47.5, 46	7.5	0.28	...
	1.0	5.0	45, 44	7.3	...	4.5
	2.0	5.0	57, 59	11.0	...	6.2
Whole Dried Liver Powder No. 1	1.0	0	48, 45	4.7	14.1	...
	2.0	0	62, 62.5, 60	10.4	15.6	...
	3.0	0	67, 70, 69.5	16.5	16.5	...
	1.0	5.0	61, 60, 64.5	9.6	...	4.9
	2.0	2.0	64, 65.5	12.3	...	1.9
Liver, Desiccated, Defatted	1.0	0	37, 37, 39	3.4	6.8	...
	2.0	0	54, 53, 55.5	7.0	7.0	...
	3.0	0	60, 62.5, 62	10.0	6.7	...
	1.0	2.0	51, 53, 50	5.8	...	2.4
	2.0	5.0	64, 62, 64	11.6	...	4.6
Yeast Autolysate No. 1	1.0	0	32, 33, 29	4.7	9.4	...
	2.0	0	52, 53.5, 53	9.0	9.0	...
	3.0	0	66, 68.5, 63	14.1	9.4	...
	1.0	5	54, 57, 59	10.1	...	5.4
	2.0	5	63, 67.5, 67	14.0	...	5.0
Yeast Autolysate No. 2	1.0	0	35, 34, 34	4.5	2.2	...
	2.0	0	66, 64.5, 64	8.8	2.2	...
	3.0	0	75, 78	11.4	1.8	...
	1.0	5	72, 72, 74	11.0	...	5.5
	2.0	5	79, 84, 81	14.5	...	5.7
Rice Bran Concentrate	1.0	0	20.5, 19, 22	2.3	11.5	...
	2.0	0	39.5, 36, 34	4.4	11.2	...
	3.0	0	50, 46.5, 47.5	7.0	11.6	...
	1.0	5	48, 53, 53	7.6	...	5.3
	2.0	5	59, 60.5, 57	10.8	...	6.4
Dried Yeast	1.0	0	19.5, 16, 19	1.8	3.6	...
	2.0	0	33, 31, 34.5	3.8	3.8	...
	3.0	0	46, 45, 51	7.0	4.6	...
	1.0	5.0	45, 45, 48.5	6.6	...	4.8
	2.0	5.0	57, 50, 55	8.6	...	5.0
Liver Concentrate Paste	1.0	0	37, 38, 36.5	3.2	3.2	...
	2.0	0	52, 49, 55.5	7.4	3.7	...
	3.0	0	59, 63, 60.5	10.8	3.6	...
	1.0	5.0	57, 57, 62.5	8.4	...	5.2
	2.0	5.0	64, 67, 61.5	12.7	...	5.4

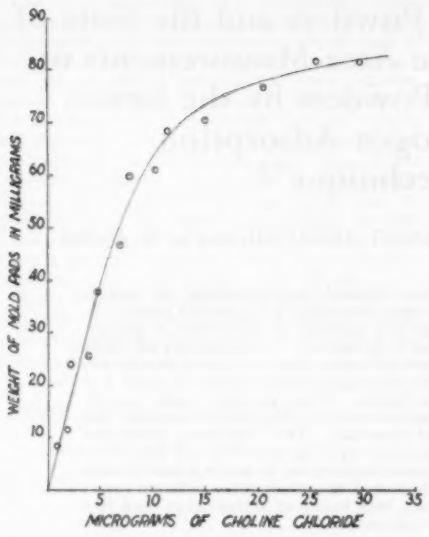

Fig. 1.

curves are not superimposable and a new standard curve must be prepared for each group of analyses. Duplicate or triplicate samples should be run on all standard and unknown solutions and an average mold weight figure should be used in constructing the standard curve or for computing the unknowns. Unknown samples may be run at several levels of choline concentration as a check. At higher concentrations, results are less accurate and may vary considerably due to linear deviation with a marked tendency toward high results. Where determinations are being carried out constantly with continued propagation of the mold, rapid or marked changes in the sensitivity to the mold are seldom noted. However, if the mold is carried on a slant and stored in a refrigerator for any length of time, it is well to carry out an orientation determination using graded amounts of choline standard solution to ascertain the intensity of the mold response to be expected and the level at which dilutions of unknowns might best be run. In a like manner, orientation determinations on unknowns should also be carried out at several levels in order to determine roughly the expected choline content and the proper dilution which will produce a concentration within the range of linear sensitivity.

The method requires rather large amounts of choline if compared in sensitivity to microbiological methods for other substances such as the vitamins. This, however, is in accord with the fact that the choline concentrations of tissues generally are of a magnitude greater than that of the vitamins. Similarly, the choline requirements of most species are far above the vitamin requirements.

The reproducibility of the method is fair when considered in the light of other microbiological procedures. Recovery of added choline is likewise satisfactory when considered statistically. Dry weights of molds from duplicate or triplicate flasks generally agree within 8% on the average with single wide variations observed from time to time. The recovery of added choline is usually within 10%. This, too, is an average figure.

TABLE II.—EFFECT OF BASE EXCHANGE ON CHOLINE VALUES FOUND

Sample	Micrograms of Choline Chloride Found without Adsorption	Micrograms of Choline Chloride Found after Adsorption
Liver Extract Injectable, 2 U. S. P. Units	5.5	5.1
	5.7	5.4
	5.6	5.1
Liver B Vitamins Injectable	4.4	4.0
	4.6	4.4
	4.9	5.0
Whole Dried Liver Powder	5.8	4.2
	6.1	4.0
	5.4	3.7
Liver Desiccated and Defatted	7.4	5.9
	7.2	5.6
	7.6	6.6
Yeast Autolysate No. 1	9.7	8.9
	9.4	8.3
	10.8	9.0
Yeast Autolysate No. 2	8.5	8.8
	9.4	8.6
	9.7	8.8
Rice Bran Concentrate	7.6	7.7
	8.4	7.5
	8.0	7.8
Dried Yeast	6.8	6.6
	6.9	6.4
	6.3	5.9
Liver Concentrate Paste	7.6	6.9
	7.3	6.3
	7.8	6.6

SUMMARY

Recent interest in choline as a therapeutic agent has created a need for methods of determination applicable to biological materials used in the pharmaceutical industry. The microbiological method based upon the growth of the cholineless mutant of the mold *Neurospora crassa* is considerably more sensitive and more specific than any of the chemical methods investigated to date. Although growth of the *Neurospora* is supported by various choline analogs, so that the reaction is not as specific as might be desired, these analogs, so far as is known, do not occur in any appreciable quantity in most biological substances. The *Neurospora* also responds to methionine but this response is slight and is not a serious deterrent in the method. Results obtained indicate that the method, while not as sensitive as many microbiological procedures, is reliable and reproducible and that added choline can be recovered with a fair degree of accuracy and consistency.

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Studies on Pharmaceutical Powders and the State of Subdivision. II. Surface Area Measurements of Some Pharmaceutical Powders by the Low-Temperature Nitrogen Adsorption Isotherm Technique*,†

By J. V. SWINTOSKY,‡ S. RIEGELMAN,§ T. HIGUCHI, and L. W. BUSSE

The practicability of the nitrogen adsorption method in determining the specific surface area of pharmaceutical powders has been illustrated in a previous paper. In this report the application of this technique to a number of nonrefractory pharmaceutical powders not previously investigated is presented. Comparisons are made of the specific surface areas of differently prepared forms of the same drug powders in order to provide a basis for interpreting the pharmacological, bacteriological, and pharmaceutical behaviors of these different forms. Other powders were investigated to extend the available quantitative data pertaining to the particle size and surface areas of some common pharmaceutical materials. The usefulness, characteristics, and limitations of this method in analysis of drug powders are discussed. Data are presented for measurements on bismuth subnitrate, bismuth subcarbonate, mercuric sulfide, and sulfanilamide. Also these studies include three different forms of sulfur, two forms of ammoniated mercury, two forms of sulfadiazine, and two forms of sulfathiazole.

THIS PAPER constitutes the second in a contemplated series of reports pertaining to studies on pharmaceutical powders and the state of subdivision. The first paper (1) described the apparatus and the method of approach followed in determining the specific surface areas of powders. In this report the application of the nitrogen adsorption (BET) method (2) to a number of pharmaceutical powders not previously investigated is presented.

The nitrogen adsorption method, because of its nature, has been applied only to a very limited extent to a few organic compounds and other nonrefractory substances. A brief summary of some of the characteristics (3) of this analytical technique serves to indicate its usefulness and limitations as applied to the analysis of pharmaceutical powders.

The method can be used for measuring the absolute surface areas of either porous or nonporous powders. Further, it can yield correct values for the surface average particle size if the material is nonporous, or if only micropores are present and their surfaces are not included in the surface area measurements. The method is restricted to solids with sufficiently large absolute areas to yield an adsorption value well in excess of the experimental errors involved. Emmett (3) suggests that a 50-cc. sample of powder should have a minimum absolute surface area of two square meters if the experimental error is not to distort the true specific surface area.

In surface area studies of refractory substances, it is customary to degas the samples at temperatures of several hundred degrees in order to remove all capillary condensed vapors and gases. With nonrefractory pharmaceutical powders, such elevated temperatures are impossible without altering the chemical and physical states of these substances. Therefore the process is necessarily restricted to much lower temperatures usually not exceeding 110°. It is unlikely that all capillaries which may be present are completely degassed at these temperatures; however, such capillaries are of little consequence in pharmacy and therapeutics, especially since they play a very minor role in determining the solubility and the rate of dissolution of ordinary crystalline materials. Indeed it would appear that surface area measurements which do not account for the capillary surfaces should correlate better with observed therapeutic and pharmaceutic effects than measurements which include these microcapillary pores.

EXPERIMENTAL

In the present investigation several typical pharmaceutical powders were studied. These were

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† This paper is based in part on a thesis submitted by Joseph V. Swintosky to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

‡ American Foundation for Pharmaceutical Education Fellow, 1947–1948.

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chosen to permit comparison of the specific surface areas of differently prepared forms of the same drugs so that the data obtained could be used in interpreting the pharmacological, bacteriological, and pharmaceutical behaviors of these different forms. Other additional powders were chosen to extend the available data pertaining to the particle size and surface areas of some common pharmaceutical powders since such quantitative data are not generally available. The choice of the powders was necessarily restricted to those which could be measured by the nitrogen adsorption method. Substances such as boric acid, for example, which are volatile and decompose even at low temperatures, could not be studied by this technique.

Eight different types of drug powders were investigated. However, since several forms of some of these drug powders were studied, a total of thirteen samples was analyzed.

EXPERIMENTAL PROCEDURE AND DATA

Bismuth Subnitrate.—Mallinckrodt U. S. P.,[1] grade bismuth subnitrate was used without pretreatment. The powder was dried over granular anhydrous magnesium perchlorate in a desiccator for at least a week, as were all other powders unless otherwise indicated, prior to adsorption measurements.

Bismuth Subcarbonate.—A U. S. P. grade of bismuth subcarbonate was used without pretreatment.

Sulfanilamide.—Merck U. S. P. grade sulfanilamide was used without pretreatment.

Sulfadiazine.—Two different samples of sulfadiazine were analyzed:

(1) Calco U. S. P. grade sulfadiazine, supplied by Smith, Kline, and French Laboratories, was used without pretreatment.

(2) A microcrystalline sulfadiazine containing approximately 2% gelatin was used without pretreatment. This material likewise was obtained from the Smith, Kline, and French Laboratories.

Sulfathiazole.—Two different samples of sulfathiazole were analyzed:

(1) Smith, Kline, and French microcrystalline sulfathiazole containing approximately 2% gelatin was used without pretreatment.

(2) International Pulverizing Corp. "micronized" sulfathiazole was used without pretreatment.

Mercuric Sulfide.—Mallinckrodt red mercury sulfide (cinnabar) was used without pretreatment.

Sulfur.—Three different samples of sulfur were analyzed:

(1) Mallinckrodt U. S. P. grade precipitated sulfur was used without pretreatment.

(2) A colloidal sulfur was prepared by reacting sodium thiosulfate with hydrochloric acid in the presence of methyl cellulose.[2] The sulfur was separated from the water-soluble components by centrifuging in a Sharples centrifuge at 50,000 r. p. m. After drying the sulfur over magnesium perchlorate in a desiccator for two weeks, the mass of sulfur was crushed with a mortar and pestle to a granular consistency prior to measurement.

(3) A Doak Co., Inc., colloidal grade sulfur was also studied. This sulfur initially was approximately 40% pure, the remainder being proteinaceous material. The powder was placed in water allowing the proteinaceous material to be dissolved, and the suspended sulfur was then conveniently removed by centrifuging at 50,000 r. p. m. in a Sharples centrifuge. The sulfur was dried over magnesium perchlorate in a desiccator for a period of two weeks and the resulting mass was crushed to a granular consistency with a mortar and pestle prior to measurement.

Ammoniated Mercury.—Two samples of ammoniated mercury were analyzed:

(1) Mallinckrodt U. S. P. grade ammoniated mercury was used without pretreatment.

(2) A colloidal ammoniated mercury was prepared by reacting solutions of mercuric chloride and ammonium hydroxide in the presence of methylcellulose. The soluble components were separated from the ammoniated mercury by centrifuging with a Sharples centrifuge. After drying the mass of ammoniated mercury over magnesium perchlorate for two weeks, the material was crushed with a mortar and pestle and then analyzed in a nitrogen adsorption apparatus.

Nitrogen adsorption isotherms for these powders were obtained with a conventional BET apparatus as described in a preceding paper (1) of this series. Temperatures and evacuation times used in these analyses are listed for each powder in the tabulated data. As can be seen in the table, degassing temperatures in all cases were below 110°. At these lower temperatures it was necessary to carry out the evacuation process for relatively longer periods of time. Even then, as previously mentioned, the process may not have been complete. However, experience in this and other laboratories has shown that the values obtained by use of these lower degassing temperatures do not differ significantly from those obtained through use of much higher temperatures when the powders are relatively nonporous.

The relative vapor pressures of the adsorbent samples ultimately determined the temperatures used during the evacuation procedures, and the pumping time was in turn influenced by the temperature of the sample during the degassing period. All deadspace measurements were made with helium gas prior to the adsorption measurements.

RESULTS AND DISCUSSION

The results of the analysis on the various powders are shown in Table I and Figs. 1–12. Adsorption hysteresis was not observed in any of these measurements, so that the powders analyzed were assumed to be nonmicroporous in nature.

The samples of bismuth subnitrate and bismuth subcarbonate showed an appreciable difference in surface area and particle size. The larger particle size of bismuth subnitrate is evident qualitatively by feeling of the two powders or by observation with a microscope. The measurements would indicate that the bismuth subnitrate is about ten times as coarse as the bismuth subcarbonate.

Sulfanilamide and its derivatives showed marked differences in particle size. U. S. P. sulfadiazine was shown to have a specific surface area about five

[1] U. S. P. grade chemicals imply U. S. P. XII or XIII.

[2] Details regarding the preparation and utilization of several of these powders in bacteriostatic studies will be reported in a forthcoming paper of this series.

TABLE I.—SURFACE AREA MEASUREMENTS OF PHARMACEUTICAL POWDERS BY LOW TEMPERATURE NITROGEN ADSORPTION METHOD

Material	Weight of Sample, Gm.	Sample Number	Analysis Number	V_m, Cc.	Area, m^2/Gm.	Average Area, m^2/Gm.	Density	Surface Average Diameter of Particle, μ
Bismuth Subnitrate (U. S. P.)	17.08	4A	1	0.136	0.59_2	0.63	4.93	1.93
	17.90		2	0.154	0.67_0			
	17.90		3	0.145	0.63_1			
Bismuth Subcarbonate (U. S. P.)	18.24	5A	1	1.272	5.53	5.56	6.86	0.157
	14.54		2	1.282	5.58			
Sulfanilamide (U. S. P.)	29.79	6A	1	0.0609	0.26_3	0.28	1.4_9[a]	14.4
	29.79		2	0.0604	0.26_2			
	31.95		3	0.0684	0.29_8			
	31.95		4	0.0641	0.27_9			
Sulfadiazine (U. S. P.)	19.22	7A	1	0.360	1.57	1.57	1.50_9[a]	2.55
	19.22		2	0.360	1.57			
Sulfadiazine (microcrystalline, S. K. F.)	1.594	8A	1	3.46	15.1	15.1	1.50_9[a]	0.265
	1.520		2	3.47	15.1			
	1.528		3	3.47	15.1			
Sulfathiazole (microcrystalline, S. K. F.)	6.240	9A	1	0.936	4.07	4.12	1.50_9[a]	0.971
	5.980		2	0.960	4.18			
Sulfathiazole (Micronized, I. P. C.)	14.88	10A	1	0.473	2.06	2.10	1.50_9[a]	1.90
	14.88		2	0.492	2.14			
Mercuric Sulfide	12.45	11A	1	0.0865	0.32_4	0.33	8.10	2.24
	38.26		2	0.0752	0.32_7			
Sulfur (U. S. P.)	20.04	12A	1	0.0594	0.25_3	0.26	2.07	11.1
	20.97		2	0.0732	0.31_8			
	20.97		3	0.0531	0.23_1			
	21.94		4	0.0538	0.23_4			
	21.94		5	0.0612	0.26_6			
Sulfur (Doak Co., Inc.)	17.47	13A	1	0.911	3.96	3.96	2.07	0.732
	17.47		2	0.911	3.96			
Sulfur (chemically pptd. and spray dried)	4.290	14A	1	0.403	1.75	1.73	2.07	1.68
	12.91		2	0.392	1.71			
Ammoniated Mercury (U. S. P.)	48.70	15A	1	0.214	0.93	0.96	5.2_1[a]	1.19
	43.74		2	0.228	0.99			
Ammoniated Mercury (chemically pptd. and spray dried)	11.56	16A	1	0.934	4.06	4.06	5.2_1[a]	0.280
	11.56		2	0.934	4.06			

[a] Densities of these compounds were obtained with the nitrogen adsorption apparatus by determining the deadspace volume of a given empty sample tube followed by a determination of the deadspace of the same sample tube containing a known weight of powder. This then indicated the volume occupied by the known weight of powder. Dividing the weight of the sample by its volume gave the density directly.

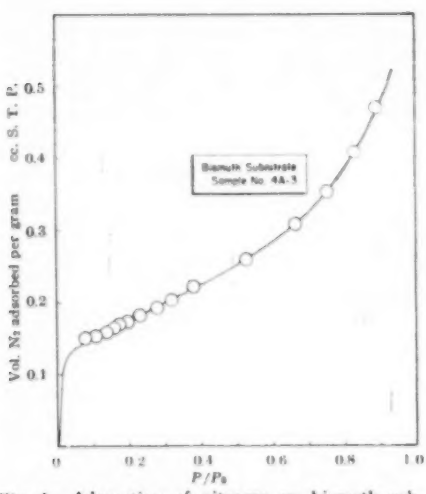

Fig. 1.—Adsorption of nitrogen on bismuth subnitrate at liquid nitrogen temperature.

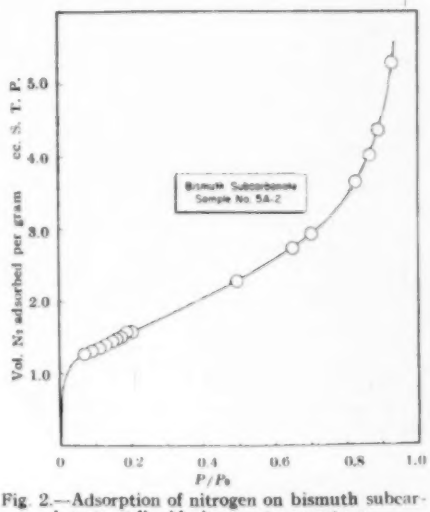

Fig. 2.—Adsorption of nitrogen on bismuth subcarbonate at liquid nitrogen temperature.

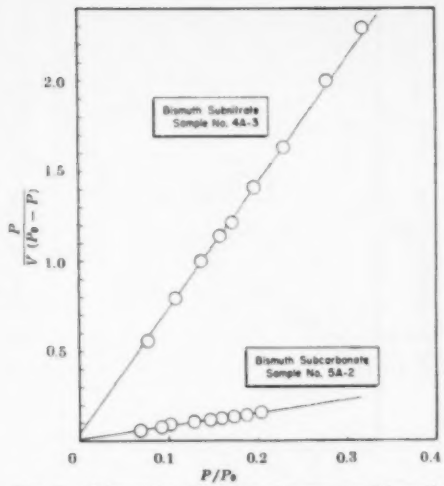

Fig. 3.—Adsorption data for nitrogen on bismuth subnitrate and bismuth subcarbonate, plotted according to the BET Equation.

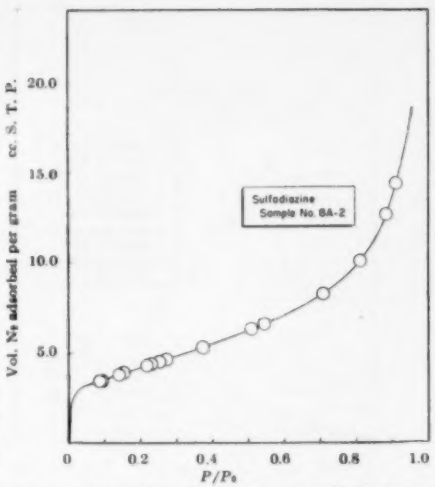

Fig. 5.—Adsorption of nitrogen on sulfadiazine at liquid nitrogen temperature.

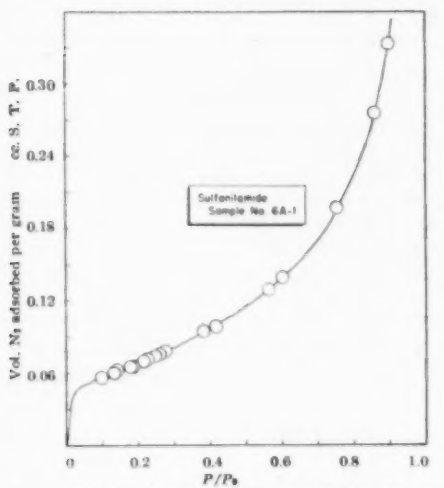

Fig. 4.—Adsorption of nitrogen on sulfanilamide at liquid nitrogen temperature.

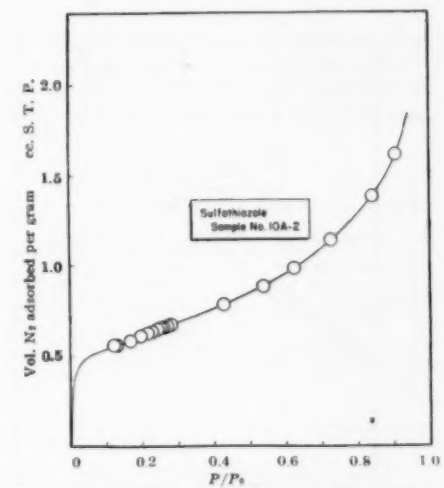

Fig. 6.—Adsorption of nitrogen on sulfathiazole at liquid nitrogen temperature.

times as great as the U. S. P. sulfanilamide, whereas the microcrystalline derivatives were considerably finer than either of the U. S. P. powders. The increased surface area of the microcrystalline derivatives is in keeping with the knowledge of increased rates of solution and absorption, and higher blood levels achieved with these substances in clinical usage (4, 5).

In the case of the sulfur powders, the U. S. P. sulfur was also shown to be extremely coarse; and it was difficult to measure accurately because of the larger relative magnitude of the deadspace error. The colloidal sulfur samples showed relatively large specific surface area values.[4] According to

[4] In the course of these measurements on the sulfur it was observed that on immersion of the sample tube in liquid nitrogen, the sulfur changed rather abruptly from a yellow to a white-colored powder. It is probable that a certain relative change in color may occur with most colored compounds being cooled to this temperature since the concentration of the excited electronic states necessary for color production would be reduced and consequently would affect the light absorption properties of the material. The mercuric sulfide likewise displayed a color change from red to orange on cooling, and, like the sulfur, also returned to its original color after being removed from the liquid nitrogen bath.

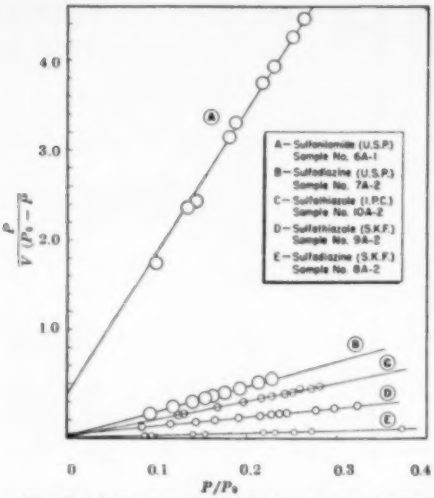

Fig. 7.—Adsorption data for nitrogen on sulfanilamide and on several grades of sulfadiazine and sulfathiazole, plotted according to the BET equation.

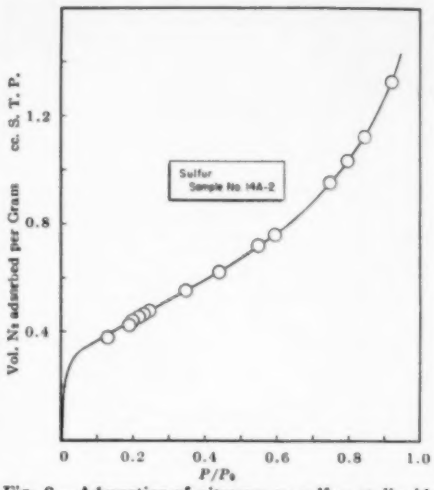

Fig. 9.—Adsorption of nitrogen on sulfur at liquid nitrogen temperature.

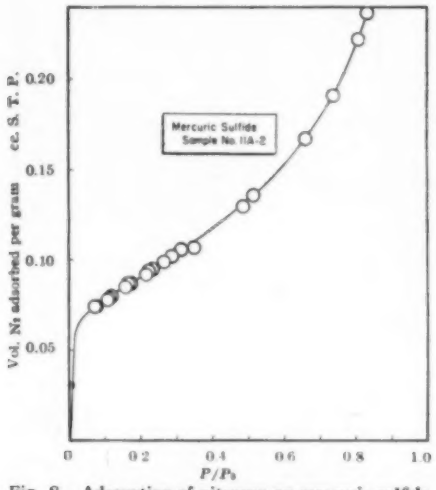

Fig. 8.—Adsorption of nitrogen on mercuric sulfide at liquid nitrogen temperature.

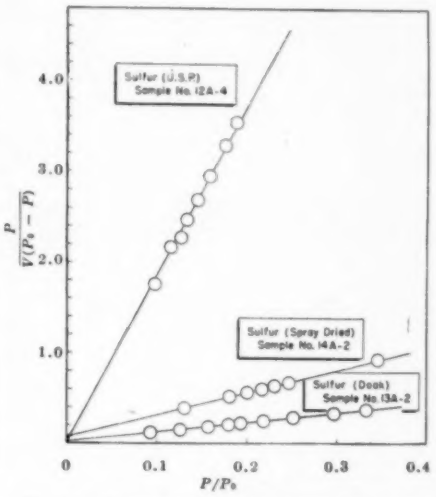

Fig. 10.—Adsorption data for nitrogen on several grades of sulfur, plotted according to the BET equation.

the hypothesis that medicinal powders with higher specific surface areas display greater solution rates and greater activities, one might assume that the efficacy of the colloidal sulfur preparations would be proportionately greater than the noncolloidal forms.

No difficulties were encountered in the measurements of the specific surface areas of the ammoniated mercury samples. The specially prepared colloidal material was shown to have a specific surface area about four times as great as the U. S. P. product.

It is apparent from the preceding data that the particle sizes and specific surface areas of different pharmaceutical powders may vary over wide ranges depending upon the composition of the material and the method of manufacture. Since it appears that the efficacy of sparingly soluble drugs may vary directly with the specific surface areas, it is evident

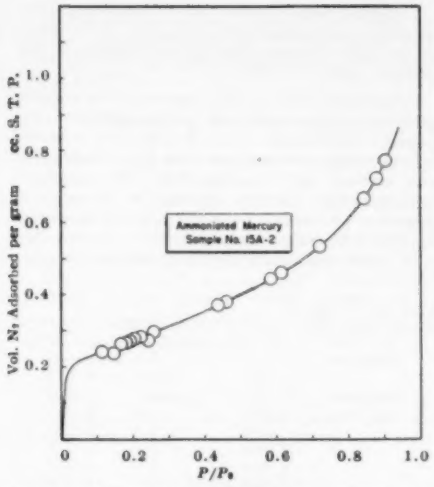

Fig. 11.—Adsorption of nitrogen on ammoniated mercury at liquid nitrogen temperature.

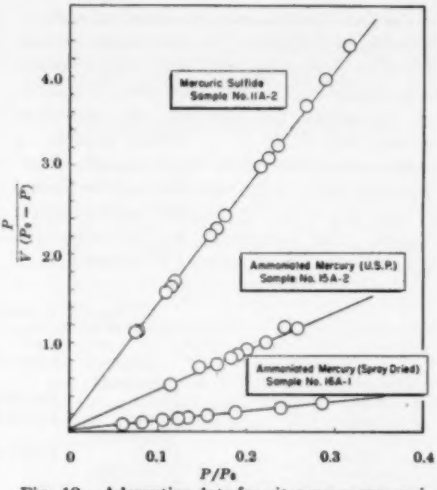

Fig. 12.—Adsorption data for nitrogen on mercuric sulfide and on two grades of ammoniated mercury, plotted according to the BET equation.

that further studies on these surface values should be made.

SUMMARY

Data are presented for specific surface area and surface average diameters for a number of pharmaceutical powders. Representative samples of these substances gave specific surface area values of 0.63 m.²/Gm. for bismuth subnitrate, 5.56 m.²/Gm. for bismuth subcarbonate, 0.28 m.²/Gm. for sulfanilamide, and 0.33 m.²/Gm. for mercuric sulfide. Three different forms of sulfur gave values of 0.26 m.²/Gm., 1.73 m.²/Gm., and 3.96 m.²/Gm., respectively. Two different forms of ammoniated mercury measured 0.96 m.²/Gm. and 4.06 m.²/Gm., respectively. Two different forms of sulfadiazine measured 1.57 m.²/Gm. and 15.1 m.²/Gm., and two different forms of sulfathiazole measured 2.10 m.²/Gm. and 4.12 m.²/Gm., respectively.

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Microscopic Characters of Some of the Crystalline Sympathomimetic Amines*

By GEORGE L. KEENAN†

MICROSCOPIC descriptions recorded in this report are the result of the study of samples of the crystalline sympathomimetic amines and their derivatives, principally furnished by Haley, and used by him in the chemical differentiation of these compounds. For convenience of reference, data on *l*-ephedrine hydrochloride and ephedrine sulfate, already on record in the literature (1) are included.

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The data here recorded are based on a determination of the optical crystallographic properties of the compounds, such data being available from a microscopic examination with the polarizing microscope and applying the immersion method (see Table I). Where possible, a similar microscopic study has been made of any crystalline addition products that could be formed by the use of the appropriate microchemical reagent. The compounds will be considered alphabetically for convenience of reference.

Refractive Indices.—α = 1.535 (commonly shown lengthwise on elongated fragments), β = indet., γ = 1.620 (shown crosswise on elongated fragments); both ±0.002.

Chloro-auric acid reagent also forms an addition product of yellow rods and needles with this compound. With crossed nicols the extinction is parallel and the sign of elongation with the selenite plate and crossed nicols is negative. In convergent polarized light (crossed nicols), no interference figures are observed since the crystals extinguish sharply. The significant refractive indices are: α = 1.568 (commonly shown lengthwise on rods),

TABLE I.—TABULATION OF DATA

Substance	Habit	Extinction	Elongation	Refractive Indices α	β	γ
d-Desoxy ephedrine HCl	Fragments	...	...	1.530	1.537	1.615
dl-Desoxy ephedrine HCl	Plates	Parallel	Negative	1.535	Indet.	1.620
l-Ephedrine HCl	Rods	Parallel	Negative	1.530	1.603	1.638
dl-Ephedrine HCl	Fragments	...	...	1.570	1.608	1.630
Ephedrine SO_4	Plates	Parallel	...	1.540	1.565	1.587
Paredrine HBr	Fragments	...	...	1.560	1.680	1.734
Tuamine SO_4	Plates	...	...	1.458(ω)	...	1.468(ε)
Vonedrine HCl	Rods	Parallel	Negative	1.577	Indet.	1.603

d-DESOXYEPHEDRINE HYDROCHLORIDE

In Ordinary Light.—The habit is not significant, the material breaking up into irregular fragments when crushed for microscopic study.

Characters Shown in Parallel Polarized Light (Crossed Nicols).—It is characteristic of the substance to show many fragments that do not extinguish sharply with crossed nicols.

Characters Shown in Convergent Polarized Light (Crossed Nicols).—The substance is biaxial and axial plane and optic axis figures are frequently observed. Optic sign (+). 2*E*, not large.

Refractive Indices.—α = 1.530, β = 1.537, γ = 1.615; all ±0.002. The β-value is frequently shown.

With chloro-auric acid, yellow rods and needles are formed, many of the rods having square ends. The rods and needles show parallel extinction with crossed nicols and with the selenite plate and crossed nicols the sign of elongation is negative. In view of the fact that the crystals extinguish sharply with crossed nicols, no interference figures are observed in convergent polarized light (crossed nicols). The significant refractive indices of this addition product are: α = 1.580 (shown lengthwise, and very common), β = indet., γ = slightly higher than the *n* for methylene iodide (1.734), but many rods match this liquid crosswise; both ±0.002.

dl-DESOXYEPHEDRINE HYDROCHLORIDE

In Ordinary Light.—The substance consists of very small, platy crystals, some of which are roughly six-sided in habit, and others elongated.

Characters Shown in Parallel Polarized Light (Crossed Nicols).—The extinction is usually parallel on elongated fragments and the sign of elongation with selenite plate and crossed nicols is negative. The rods and needles invariably extinguish sharply.

Characters Shown in Convergent Polarized Light (Crossed Nicols).—No interference figures observed. β = indet., γ = >1.734 (crosswise on rods). β is probably shown on plates tipped on edge.

l-EPHEDRINE HYDROCHLORIDE

In Ordinary Light.—The habit is elongated prisms and rods. It probably crystallizes in the orthorhombic system.

Characters Shown in Parallel Polarized Light (Crossed Nicols).—The extinction is parallel and the sign of elongation with the selenite plate and crossed nicols is negative.

Refractive Indices.—α = 1.530, β = 1.603, γ = 1.638 (1).

dl-EPHEDRINE HYDROCHLORIDE

In Ordinary Light.—The habit is not significant the substance breaking up into irregular fragments.

Characters Shown in Parallel Polarized Light (Crossed Nicols).—Most of the fragments extinguish sharply with crossed nicols and occasionally some remain bright under the same conditions.

Characters Shown in Convergent Polarized Light (Crossed Nicols).—The substance is biaxial and occasionally optic axis figures are observed. Optic sign (−).

Refractive Indices.—α = 1.570 (common), β = 1.608, γ = 1.630 (also common); all ±0.002.

EPHEDRINE SULFATE

In Ordinary Light.—The habit is hexagonal plates.

Characters Shown in Parallel Polarized Light (Crossed Nicols).—The extinction is parallel on elongated forms.

Characters Shown in Convergent Polarized Light (Crossed Nicols).—No interference figures are shown. Optic sign (+). 2E is large.

Refractive Indices.—α = 1.540, β = 1.565, γ = 1.587. Plates extinguishing sharply show index 1.540 in one extinction position and 1.565 in the other (1, 2).

PAREDRINE HYDROBROMIDE

In Ordinary Light.—The habit is largely irregular fragments without significant form.

Characters Shown in Parallel Polarized Light (Crossed Nicols).—Some fragments extinguish sharply with crossed nicols while others remain bright when the microscope stage is revolved.

Characters Shown in Convergent Polarized Light (Crossed Nicols).—Partial biaxial interference figures are frequently observed, these showing one optic axis in the microscopic field. Optic sign (−).

Refractive Indices.—α = 1.560, β = 1.680, γ = 1.734; all ±0.002.

A solution of paredrine hydrochloride becomes blood-red on the addition of chloro-auric acid reagent.

TUAMINE SULFATE

In Ordinary Light.—The substance consists of colorless, irregularly shaped plates and fibrous flakes.

Characters Shown in Parallel Polarized Light (Crossed Nicols).—The larger, irregular plates usually do not extinguish sharply when the microscope stage is revolved. Birefringence not strong but moderate.

Characters Shown in Convergent Polarized Light (Crossed Nicols).—The larger, irregular plates not extinguishing sharply show a distinct uniaxial interference figure with positive optic sign. These figures occur quite frequently.

Refractive Indices.—ω = 1.458, ϵ = 1.468; both ±0.002.

The rods and needles formed with picric acid show inclined extinction and characteristic anomalous polarization colors with crossed nicols, the colors consisting of peculiar blues and purples.

VONEDRINE HYDROCHLORIDE

In Ordinary Light.—The substance consists of small, rod-like fragments.

Characters Shown in Parallel Polarized Light (Crossed Nicols).—The extinction is parallel and the sign of elongation is negative on the elongated fragments.

Characters Shown in Convergent Polarized Light (Crossed Nicols).—No interference figures observed.

Refractive Indices.—α = 1.577 (commonly shown lengthwise), β = indet., γ = 1.603 (commonly shown crosswise); both ±0.002.

The chains of prisms which the compound forms with chloro-auric acid are yellow. The prisms vary in habit and are probably monoclinic. Prisms showing inclined extinction and the maximum double refraction have a minimum refractive index (α) of 1.603 and a maximum value (γ) of 1.734; both ±0.002 (2).

SUMMARY

The principal microscopic characters of some of the crystalline sympathomimetic amines have been determined by the immersion method and suitable microchemical reagents, the optical crystallographic data being recorded in Table I.

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Effect of Metabolite Antagonists on Histidine Decarboxylase*

By J. M. BEILER, ROBERT BRENDEL, MIRIAM GRAFF, and GUSTAV, J. MARTIN

The results of a study of histidine decarboxylase metabolite antagonists are reported. The compounds used in the study included a series of vitamin antagonists, compounds containing phenolic hydroxyl groups and compounds structurally related to histidine.

SOME ten years ago Werle (1, 2) and Holtz (3) reported the existence in animal tissues of an enzyme capable of decarboxylating histidine to form histamine. This they called histidine decarboxylase. The decarboxylation was slow, and the amount of CO_2 formed was too small to be measured in the Warburg, so the reaction was studied pharmacologically.

* Received Nov. 27, 1948, from the Research Laboratories The National Drug Co., Philadelphia, Pa.

EXPERIMENTAL

The procedure used was a modification of that of Werle and Herrmann (2). Two grams of guinea-pig kidney (fresh weight) were ground with sand in 4 cc. of distilled water. The mixture was centrifuged and 1 cc. of the supernatant was added to 2.5 cc. of

M/15 phosphate buffer *p*H 8.5, containing 10 mg. of *l*-histidine. One and seven-tenths cubic centimeters of this solution was placed in the Warburg cups in which the experiment was run, and 0.3 cc. of a neutral solution of the substance whose effect was being determined was added to this. The mixture was shaken at 37° under N_2 for two to three hours, and the amount of histamine formed was determined by comparing the amplitude of contraction produced in the isolated guinea-pig intestine with that produced by known quantities of histamine. The amount of histamine formed in the reaction was quite constant, and varied from 3 to 4γ/cc.

Preliminary experiments had shown that the tissue extract alone, and the *l*-histidine alone, produced no contraction of the guinea-pig intestine, so that these were not run routinely as controls. Controls were run with tissue extract and histidine, and tissue extract without histidine plus the compound being tested. In cases where a stimulation or inhibition was obtained, an additional control was run with tissue extract plus the compound being tested plus a measured amount of histamine. This showed whether the effect noted was due to an action of the compound being tested on histidine decarboxylase or to an action on the response of the guinea-pig intestine to histamine.

RESULTS

The effects of three series of compounds on the action of histidine decarboxylase were studied.

(A) A series of vitamin antagonists was investigated. This was done on the premise that, should some vitamin function as the coenzyme of the decarboxylase, its displacement by a suitable antagonist should result in an inhibition of the enzyme.

(B) Werle (4) showed that the enzyme was inhibited by adrenaline, dopa, and several other compounds of like structure. This he ascribed to the presence in the various molecules of phenolic hydroxyl groups. Accordingly, a number of compounds containing phenolic hydroxyl groups were studied.

(C) A number of compounds whose structures possessed varying degrees of similarity to that of histidine were investigated. The object here was to determine whether an inhibition of the enzyme action could be achieved by a displacement of the substrate.

The results are listed in Tables I, II, and III. Inhibitions were calculated by comparing the amounts of histamine formed in the controls (histidine and tissue extract) with those formed in the presence of added amounts of the compounds being tested.

TABLE I.—EFFECT OF VITAMIN ANTAGONISTS ON THE ACTION OF HISTIDINE DECARBOXYLASE

Compound	Concentration (Mg./Cc.)	Inhibition, %
Glucoascorbic Acid	1.0	0
Isoriboflavin	1.0	0
Desthiobiotin	1.0	0
Pantoyl taurine	1.0	0
Pyrithiamine	1.0	Stimulation
7-Methyl Folic Acid	1.0	Stimulation
Desoxypyridoxine	1.0	0
Pyridine β-sulfonamide	1.0	0

TABLE II.—EFFECT OF PHENOLIC COMPOUNDS ON THE ACTION OF HISTIDINE DECARBOXYLASE

Compound	Inhibition, % 1.0 Mg./Cc.	0.1 Mg./Cc.	0.01 Mg./Cc.
Catechol	100	40	..
Dopa	100	25	..
N-methyl dopa	40	..	..
Salicylic Acid	20	..	..
Tyrosine	0	..	..
N-methyl tyrosine	0	..	..
Phenylalanine	0	..	..
p-Fluorophenylalanine	0	..	..
Benzoquinone	100	80	0
Inositol	0	..	..

TABLE III.—EFFECT OF COMPOUNDS STRUCTURALLY RELATED TO HISTIDINE ON THE ACTION OF HISTIDINE DECARBOXYLASE

Compound	Inhibition, % 2.5 Mg./Cc.	1.0 Mg./Cc.
Imidazoleamino methyl sulfonic acid	35	0
N-Sulfanilyl-4-aminobenzimidazole	..	20
Benzoxazolone	..	20
3-Benzothiophene α-amino propionic acid	..	25
β-2-thienyl alanine	..	40

DISCUSSION

The results show that none of the vitamin antagonists tested produced any inhibition of the action of histidine decarboxylase. Three explanations may be offered for this finding. First, the enzyme may be associated with a coenzyme which is analogous to none of the compounds tested. Second, there may be no coenzyme. Or, finally, the coenzyme may be so tightly bound to the enzyme that it cannot be displaced by the methods used. Either of the last two possibilities is consistent with the observation of Werle (5) that the enzyme does not lose activity on dialysis.

No explanation is readily forthcoming for the fact that both pyrithiamine and 7-methyl folic acid were found actually to stimulate to a slight extent the action of histidine decarboxylase. It is possible that there is some other enzyme present in the tissue extract which attacks histidine, and that this is inhibited by the vitamin antagonists in question. This would, in effect, raise the concentration of histidine available to the histidine decarboxylase, and thus increase the activity of the enzyme. Some support for this view is provided by Werle's observation (4) that thiamine actually inhibited the action of histidine decarboxylase. Folic acid, on the other hand, was tested and was found to be without effect.

The effect of phenols on the decarboxylase is striking; as may be seen from Table II, some of these were the most effective compounds tested. The extent of the inhibition caused by these phenolic compounds was dependent on the number of hydroxyl groups present, at least within the limits of the compounds tested. The effect seems to be peculiar to phenolic hydroxyls; inositol, a compound rich in nonphenolic hydroxyls, was completely inactive.

The extent of the inhibition varied with the number of phenolic hydroxyls present, at least within the limits of the compounds tried; all the compounds

with two hydroxyl groups were more active than those with only one. The effectiveness of the compounds varied inversely with the complexity of the side chain; catechol was more active than dopa, which in turn was much more active than N-methyl dopa; salicylic acid was more active than tyrosine. Whether the action of benzoquinone is analogous to that of the phenolic compounds is not known.

The third group tested comprised a number of compounds structurally related to histidine. The results indicate that there is no rigid specificity here; compounds of quite diverse structures were effective as inhibitors. The effect was small, being much less than that produced by some of the phenolic compounds, and it may be that this is a measure of the failure of any of the compounds tested to represent what would be a truly specific displacer structure.

SUMMARY

Three series of compounds have been tested to determine their effect on the action of histidine decarboxylase. These included vitamin antagonists, compounds containing phenolic hydroxyl groups, and compounds structurally related to histidine.

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The Oxytocic Evaluation of Posterior Pituitary Solutions, U. S. P., the Guinea-Pig Uterus*,†

By CLARENCE E. POWELL and EDWARD E. SWANSON

Several methods for evaluating the oxytocic potency in posterior pituitary solutions are discussed. Experiments were carried out in which the method for the blood pressure response in chickens was applied to the Official U. S. P. Guinea-Pig Uterus Method with certain changes.

THE PRESENT METHOD of evaluating the oxytocic potency in posterior pituitary solutions, as described in the U. S. P. XII and XIII, has many disadvantages. Morrell (1) enumerated the difficulties encountered with this procedure and suggested a method based on the quantal response of a single guinea-pig uterus, divided into 8 strips in a common bath with separate levers. With an all or none response, it was shown that the error of each individual test could be computed independently and the potency estimated in terms of a standard from regression lines.

Smith and Vos (2) have designed a method for the chicken's blood pressure response to pituitary preparations, first described by Coon (3). The potency and experimental error were computed mathematically. Later, Vos (4) proposed the same method of calculation for estimating the potency of ergonovine on the rabbit's uterus. By this method, the standard doses remained constant and the unknown doses varied in size, so that some gave shorter or equal latent periods and others, longer latent periods. Thompson (5) further investigated this design on the chicken's blood pressure response to pituitary solutions and reported results that were in close agreement with the official uterine method. This same design was also applied by Thompson to two tests on the official guinea-pig uterus method. His results indicated close agreement with the official assay.

Thompson, however, found a significant difference between the potency on the blood pressure of chickens and the potency on the guinea-pig uterus in one out of 17 parallel determinations, and suggested that the blood pressure method cannot always be relied upon to estimate the oxytocic activity. More recently, Holton (6) reported a statistical method of standardization of posterior pituitary extract on the isolated uterus of the rat.

In view of the advantages claimed for the chicken's blood pressure method in evaluating the oxytocic principle of posterior pituitary solutions, it appeared worth while to investigate further the guinea-pig uterus method. We have, therefore, applied the experimental design described by Vos to certain changes of the Official U. S. P. Guinea-Pig Uterus Method.

METHOD

The apparatus was the same as that used for the official method—namely, a constant temperature

* Received Dec. 31, 1948, from the Lilly Research Laboratories, Indianapolis 6, Ind.
† The authors are indebted to Lester Le Compte, Jr., for assistance in testing and computing the results of this investigation, and to Dr. Lloyd Miller, chairman of the U. S. P. Bioassay Subcommittee, for his valuable suggestions and criticisms.

bath with a 100-cc. capacity muscle chamber. Recording of uterine movements was made in the usual manner with either a gravity or a frontal writing lever, having a ratio of 1:3. Van Dyke and Hasting's solution was used for a bathing fluid. In brief, this bathing solution was prepared as described by Morrell, Allmark and Bachinski (1), page 441, paragraphs 2, 3, and 4. With this procedure the necessary *p*H of 7.4 to 7.5 can be maintained throughout the test. Standard pituitary dilutions were made up in saline solution to contain 0.02 unit per 1 cc. The unknown dilutions were made up in various strengths with saline solution.

Nonpregnant guinea pigs weighing between 200 and 350 Gm. were used. The animal was killed by a blow on the head or decapitated and the entire uterus body immediately removed. The two horns were divided at the bifurcation and immersed in van Dyke–Hastings solution, contained in a shallow dish. If large, the horns may be divided longitudinally.

Before a trial dose of the standard pituitary solution was given, the uterus was acclimated to the bathing fluid for twenty to thirty minutes to insure complete relaxation. Two or three changes of the bathing fluid aided in adjustment of the uterus. When the uterus had relaxed to a constant base line, a 0.02-unit dose of the standard solution was tried. The bathing fluid was changed when the peak of the contraction was reached. When the muscle was completely relaxed, a second 0.02-unit dose was tried. Usually ten-minute intervals between doses were taken throughout the assay. If the two standard contractions were of the same height and appeared to be maximum, decreasingly smaller doses were tried until a definite submaximal dose had been established. This submaximal dose was maintained throughout the test.

Following the determination of the submaximal dose of the standard pituitary solution, an equal volume of the unknown was tried. The height of contraction of this dose determined the subsequent unknown volumes to be given. If, however, the unknown response was above the standard contraction, smaller volumes of the unknown were used until the contractions were equal to or below the standard contraction. Conversely, if the unknown response was below the standard, larger volumes of the unknown were given equal to and above the standard responses.

A total of 13 doses, 7 standards and 6 unknowns, were found to give reliable results. The final in all cases was the standard. When the test had been completed, the heights of contractions of the standard and unknown doses were measured to the nearest 0.5 mm. and the data treated as described by Vos. A typical calculation is shown in Table I. This procedure has been used in our laboratory for the past five years and compared with the official U. S. P. method.

The results found in fourteen assays out of more than one hundred are outlined in Table II.

TABLE I.—CALCULATION SHOWING RESPONSE OF GUINEA-PIG UTERUS TO POSTERIOR PITUITARY EXTRACT

Date: June 28, 1948
Compound: Fit. Ext.
Serial No.: 12
Animal or Muscle: Guinea-Pig Uterus

Dose No.	Standard Log Dose	Log Response	Interpolate	Unknown Log Dose	Log Response	x	y
1	0.602	0.839	...	...	...	...	...
2	...	...	0.839	0.602	0.833	0.000	0.006
3	0.602	0.839	...	...	...	...	...
4	...	...	0.857	0.544	0.851	0.058	0.006
5	0.602	0.875	...	...	...	...	...
6	...	...	0.884	0.602	0.887	0.000	−0.003
7	0.602	0.892	...	...	...	...	...
8	...	...	0.898	0.653	0.909	−0.051	−0.011
9	0.602	0.903	...	...	...	...	...
10	...	...	0.909	0.544	0.903	0.058	0.006
11	0.602	0.914	...	...	...	...	...
12	...	...	0.914	0.653	0.919	−0.051	−0.005
13	0.602	0.914	...	...	...	...	...

M = 0.003637 = 100.8%
Standard Error = 3.07%
Actual Potency = 100%

TABLE II.—RESULTS OF COMPARATIVE ASSAYS BETWEEN THE OFFICIAL U. S. P. METHOD AND THE VOS DESIGN

Sample No.	U. S. P. Assay, %	Vos Assay, % ± S. E.
1	100	104.10 ± 4.30
2	120	118.00 ± 14.86
3	100	85.61 ± 12.72
4	100	90.20 ± 6.56
5	100	94.01 ± 6.05
6	100	104.30 ± 10.77
7	100	93.26 ± 3.19
8	100	96.54 ± 3.97
9	80	80.74 ± 4.86
10	80	89.69 ± 5.53
11	80	83.63 ± 3.30
12	100	100.80 ± 3.07
13	110	112.80 ± 1.93
14	90	90.03 ± 6.63

SUMMARY

1. A method for the evaluation of the oxytocic principle in posterior pituitary preparations has been described.

2. The method requires a simple constant temperature bath and the use of uteri from guinea pigs weighing between 200 and 350 Gm.

3. The method utilizes the Vos design for conducting the test and for computation of potency and standard error.

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Fluorometric Determination of Ketotetrahydropyridines. An Improved Method for Urine*,†

By ELMER De RITTER, FRED W. JAHNS, and SAUL H. RUBIN

An ether extraction procedure has been developed for the fluorometric determination in urine of 3,3-diethyl-2,4-diketotetrahydropyridine. By increasing the salt content of the urine, to promote "salting out" of the drug, and by buffering with borate at *p*H 9–9.5, quantitative extraction is achieved, while considerably less nonspecific fluorescence is carried along than in the direct method previously reported. Sensitivity and precision are thus increased. The method has been applied successfully, with good reproducibility and good recoveries, to urines from normal subjects and from patients with renal and hepatic disease at excretion levels as low as 1 to 2 mg. per day.

THE USE of the drug, 3,3-diethyl-2,4-diketotetrahydropyridine, Presidon,[1] as a sedative-hypnotic (1–5) has stimulated work on assay methods. In an earlier paper from this laboratory (6), a method for urine assay was described, which was based on measurement of the fluorescence of a direct dilution in alkaline, alcoholic solution, before and after quenching with hydroxylamine. Though suitable for most clinical studies, this method was not as sensitive as desired because of the presence in urine of considerable, nonspecific fluorescent substances. Also, in occasional pathological urines, hydroxylamine has been found to quench appreciable amounts of interfering fluorescence so that high "apparent drug" values are obtained.

Although an adequate correction for the amount of nonspecific fluorescence quenched by hydroxylamine can normally be made by measuring excretion of "apparent drug" in a control period before administration of the drug, it has been found much simpler and more direct to eliminate much of this interference by extraction of Presidon from urine with diethyl ether. With this modification both the sensitivity and the precision of the method have been increased and the need for an internal standard eliminated.

* Received Jan. 24, 1949, from the Nutrition Laboratories, Hoffmann-La Roche, Inc., Nutley, N. J.
† We wish to thank Dr. Saul Solomon of the Bellevue Hospital, New York City, for specimens of urine from patients with hepatic and renal dysfunction.
[1] This drug was formerly referred to as Persedon (5–7).

DESCRIPTION OF METHOD

Extraction.—Pour about 23 ml. of 25% aqueous potassium chloride into a 125-ml. separatory funnel. Pipette 1.00 ml. of urine, or an aliquot containing at least 5 γ of Presidon, and 1.00 ml. of a saturated solution of sodium tetraborate into the funnel. Extract with three successive 25-ml. portions of diethyl ether, shaking vigorously for two minutes for each extraction. If an emulsion forms, transfer the entire contents of the separatory funnel to a stoppered centrifuge tube, centrifuge briefly to break the emulsion; transfer quantitatively back to the separatory funnel for separation. Combine the three ether extracts in a 250-ml. Erlenmeyer flask, add 1.0 ml. of a 1% aqueous solution of ascorbic acid, and boil off the ether on a steam bath. To prevent bumping, add a small, alundum antibump stone or carry out the ether evaporation under a gentle stream of nitrogen. Remove the last traces of ether under a stream of nitrogen and avoid overheating of the residual aqueous solution. Dilute with water to a Presidon concentration of approximately 1 γ per ml., using at least 10 ml. of water.

Fluorometry.—Pipette 5.0 ml. of diluted extract and 10.0 ml. of ethanol into duplicate glass-stoppered tubes. To one of the tubes add 1.00 ml. of water and to the other 1.00 ml. of a 10% aqueous solution of hydroxylamine hydrochloride. Then add 1.00 ml. of 10% aqueous sodium hydroxide to both tubes. Set up a similar pair of tubes with 5.0 ml. of a standard solution containing 1.00 γ of Presidon per ml. in place of the 5.0 ml. of urine extract.

Stopper all tubes and mix well. Loosen but do not remove the stoppers of the tubes containing hydroxylamine and immerse these tubes in a wire rack in a constant-temperature bath at 75–80° for twenty minutes. At the end of the heating period, remove the tubes and cool to room temperature in a cold water bath.

Take fluorescence readings of the unheated and the hydroxylamine treated standard and unknown with the same filters as used for thiochrome measurements (the primary ultraviolet filter having a peak of 3700 Å. and the secondary filters a peak of 4600 Å.). Use a fluorescent glass block to set the fluorophotometer to a suitable galvanometer response just prior to each reading.

Calculation:

If S = fluorometric reading of untreated standard
S_B = fluorometric reading hydroxylamine-treated standard
U = fluorometric reading of untreated urine solution
U_B = fluorometric reading hydroxylamine-treated urine solution
A = ml. of urine extracted
D = ml. to which urine extract is diluted
V = total volume of 24-hour urine in ml.

Then

$$\frac{U - U_B}{S - SB} \times \frac{D \times V}{1000 \times A} =$$

milligrams of Presidon/day

EXPERIMENTAL

Recovery of Presidon.—The completeness of recovery by this procedure of Presidon extracted from aqueous solutions and from urines containing known added amounts of Presidon, is shown in Table I. In the case of the urines, corrections have been made for the slight quenching of nonspecific fluorescence by hydroxylamine as determined by parallel assay of urines to which no Presidon was added.

As a further check on the completeness of extraction of Presidon from urine, a series of urines with and without 25 γ of added Presidon per ml. were extracted and the fluorescence of the residual aqueous phases read in the usual manner after adding 10 ml. of ethanol, 1.00 ml. of 10% sodium hydroxide, and 1.00 ml. of water to 5.0 ml. of each solution. The comparative fluorometric readings are shown in Table II. The lack of significant differences between urines with and without added Presidon indicates that no Presidon remains in the aqueous phase after three ether extractions. Unextracted urines under these conditions showed a difference of approximately 50 galvanometer divisions due to the added Presidon.

TABLE I.—RECOVERY OF PRESIDON AT VARIOUS CONCENTRATIONS

Presidon Concentration, γ/Ml.	Per Cent Recovery	
	Water Solution	Urine
1	95	95
5	93	94
10	96	101
25	100	99
50	99	98

TABLE II.—RESIDUAL FLUORESCENCE OF AQUEOUS PHASE AFTER EXTRACTION OF URINES WITH AND WITHOUT ADDED PRESIDON

Urine Sample	Fluorometric Reading	
	No Presidon	25 γ of Presidon per Ml.
W. H.	16	15
H. M.	70	70
G. S.	14	13
M. D.	58	58
T. L.	92	97

Comparisons with Direct Method.—Comparative assays of urines without Presidon were carried out by the present extraction procedure and the direct method (6) to determine the relative magnitude of the blank fluorescence readings and of the "apparent drug" excretions. Table III gives a comparison of fluorescence blanks obtained with both methods at the same over-all dilution, namely 1.00 ml. of urine to 170 ml. In each case the reagent blank was subtracted from the observed reading. These typical data show the effectiveness of ether extraction in eliminating practically all of the blank fluorescence.

The extent of the quenching of non-Presidon fluorescence by hydroxylamine is given in Table IV for both methods. It is obvious that a considerable reduction in the "apparent drug" values is achieved by the present method.

TABLE III.—COMPARISON OF FLUOROMETRIC BLANKS BY DIRECT AND ETHER EXTRACTION METHODS

Urine Sample	Blank Fluorescence Reading	
	Direct Method (6)	Ether Extraction Method
T. L.	31	0.5
M. D.	16	1.5
G. S.	6	0.5
H. M.	24	1.0
W. H.	5	0.0
L. C.	10	1.5
W. M.	4	0.0

TABLE IV.—QUENCHING OF NONSPECIFIC FLUORESCENCE BY HYDROXYLAMINE DIRECT VS. ETHER EXTRACTION METHOD

Subject	Type[a]	Apparent Presidon, Mg./Day	
		Direct Method (6)	Ether Extraction Method
L. D.	N	2.0	0.0
F. J.	N	1.2	0.2
J. B.	N	2.4	0.4
S. R.	N	1.1	0.0
G. S.	H	5.1	0.0
M. M.	H	26.8	2.7
W. H.	R	8.7	1.8
W. M.	R	9.9	0.6
M. M.	R	17.5	4.3
T. L.	R	18.7	0.0

[a] N = normal; H = hepatic dysfunction; R = renal dysfunction.

In contrast to the direct method, in which an internal standard is obligatory, the diluted ether extracts in the present method have been found to cause no quenching of Presidon fluorescence and, hence, do not require an internal standard. Presidon extracted from aqueous solution and from urines has been found to be stable in the ether solution for at least three hours at room temperature.

DISCUSSION

Since Kubli and Schmid (7) reported that Presidon exhibits an intense blue fluorescence in strongly alkaline solution under ultraviolet light, several fluorometric assay procedures have been devised (5–8). Kubli (8) suggested ether extraction of undiluted urine after addition of sodium bicarbonate to minimize extraction of interfering fluorescent compounds. In this laboratory, however, that procedure frequently caused persistent emulsions and yielded consistently low and variable Presidon recoveries. Wilbrandt and Jaeger (5) also were unable to achieve quantitative extraction of Presidon by repeated shaking out with ether from urine diluted with an equal volume of borate buffer (*p*H 9.2). They adopted the expedient of using a single shake-out with ether, which gave about 45% recovery in the ether extract under the specified conditions. Since a quantitative extraction is inherently more reliable than an equilibrium distribution, the present method was devised to achieve this result, while retaining the advantages of the ether extraction, such as greater sensitivity, decreased interference by nonspecific fluorescence, and elimination of the need for an internal standard. We have, however, adopted the borate buffer used by Wilbrandt and Jaeger, in preference to bicarbonate as used by Kubli and Schmid. The use of borate, in conjunction with heavy salt addition to the urine, has been effective in promoting quantitative extraction with ether without troublesome emulsions.

In extensive use of this procedure for assay of normal and pathological urines, emulsion formation was encountered in less than 20% of the urine samples. A brief centrifugation was sufficient to break such emulsions in all cases.

The selection of reagents, particularly ether, which will not cause excessive fluorescence blanks, is an important consideration. Mallinckrodt's Analytical Reagent grade of anhydrous ether has been found satisfactory. Denatured alcohol (2B) has been found to be equally as good as U. S. P. ethanol. If high reagent blanks are encountered, redistillation of solvents is necessary.

The addition of ascorbic acid to the ether extract before evaporation of the ether is recommended to prevent slight losses of Presidon, which occur in the absence of ascorbic acid. The destruction is probably oxidative, possibly due to traces of peroxides in the ether. In the earlier procedure from this laboratory (6), urine solutions were heated with and without hydroxylamine before fluorometry, but in the present procedure only the blank tubes containing hydroxylamine are heated in order to avoid the possibility of further oxidative losses of Presidon in the nontreated tubes. Since the amounts of extraneous fluorescence are usually small in the ether extracts, this has little effect on the "apparent drug" values.

Satisfactory recoveries of as little as 1 γ of Presidon added to 5 ml. of urine (corresponding to a daily excretion of 0.2 to 0.4 mg. per day) have been achieved by the present method. The sensitivity and precision obtainable in an excretion study, however, are more dependent on the day-to-day variations in the "apparent drug" excretions. Since these values are relatively small, as shown by the typical data in Table IV, and the average variation from day to day is usually less than 0.4 mg., it is possible to measure Presidon excretions of 1 to 2 mg. per day with good precision.

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Determination of Heavy Metals in Somewhat Colored Solutions*,†

By E. A. McARTHUR

A new method is described for determining heavy metals. It embraces the compensation of color in the solution by multiple compartment observation tubes. Some applications, precautions, and limitations are discussed.

SINCE the official methods for the estimation of heavy metals are dependent upon the evaluation of the amount of colored sulfide formed, any color inherent in the solution of the unknown itself presents a rather difficult problem in the determination.

The British Pharmacopoeia has attempted to circumvent this difficulty by addition of dilutions of caramel to the lead standard, thus equalizing the color shown by the sample. This treatment has met with only limited success inasmuch as the color of a given unknown may or may not be of a comparable quality with that of caramel.

Introduction of a smaller amount of the sample to the lead standard has also been resorted to by

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the British Pharmacopœia, the estimation of the heavy metals content being based on the difference in the quantity of substance represented in the test and that in the standard. Rosin (1) likewise made use of this technique in his text. This approach does not actually compensate for the color of a given sample. It appears rather to be an attempt to unify the chemical constituents of the standard and unknown solutions to act as a background for the development of the test.

Examination of the constituents of the ash from an organic compound has been resorted to in some instances by the United States Pharmacopœia where color interferes in the application of the official method. Here again the procedure is lengthy and results may be seriously misleading. Ignition temperatures certainly would volatilize mercury compounds; lead, copper, and some other metals no doubt would be lost in varying degrees depending upon the intensity of heat applied in incineration.

The method herewith presented embraces the compensation of color in the unknown by utilization of dual compartment observation tubes. Thus, the specific color of the sample under test is represented with the standards in making the measurement.

METHOD

Two observation units, each consisting of two Nessler tubes of 50 ml. and 100 ml. capacity, are used. These are designated as *A* (50 ml. tube = A_1 and 100 ml. tube = A_2) and *B* (50 ml. tube = B_1 and 100 ml. tube = B_2) in Fig. 1. The 50-ml. tube is suspended in its respective 100-ml. tube by a firm fitting but adjustable rubber collar of about 1/4-inch thickness cut from a rubber stopper of No. 7 or No. 8 size. This rubber ring or collar in normal practice will be near the top of the smaller tube and, when the unit is assembled, will rest upon the top of the larger tube, thus forming a dependable support for the former. For ease of manipulation, these rings are wetted with water just prior to the beginning of a determination. The entire length of walls of both large tubes (A_2 and B_2) should be given an opaque covering, preferably white paper, to prevent interference by light from outside sources.

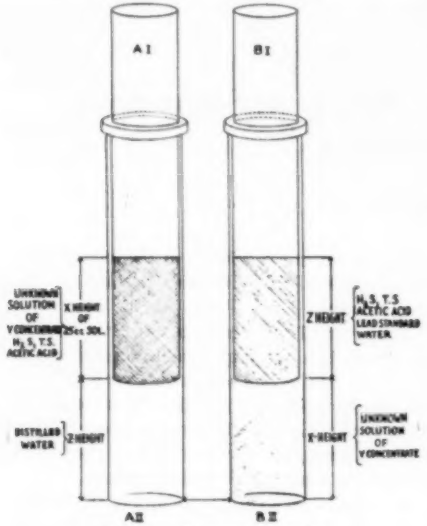

Fig. 1.—Observation units.

The unknown is processed in the observation unit *A* while the standard is developed in unit *B*. Prepare 75 ml. of solution of the unknown of such a concentration that 25 ml. will contain the quantity of sample desired for the test. Into tube A_1 introduce 25 ml. of this solution and then add the remainder to tube B_2 until a depth of about 1/4 inch greater than in A_1 has been reached. To tube A_2 add distilled water to a level identical with that of the liquid in B_2. Lower tube A_1 into A_2 by adjusting the rubber collar on A_1 so that the end of the smaller tube is just submerged in the distilled water contained in the larger tube. Now add the volume of lead standard desired to tube B_1 and sufficient distilled water to make a total of 25 ml.; insert this tube then into position in B_2. Acidify the contents of tubes A_1 and B_1 each with 1 ml. of acetic acid and mix contents thoroughly. Adjust the superimposed tube B_1 until its lower end is immersed in the liquid in B_2 and so that the color when viewed from above exactly matches that in the *A* unit observed under the same technique. (It may be necessary to tilt each unit sidewise, to free any entrapped air beneath the smaller tube, before making the above equalization.) Quickly introduce into each of A_1 and B_1, 10 ml. of hydrogen sulfide T.S. and mix the contents with glass rods. Allow to stand for ten minutes, at the end of which time comparisons may be made. These directions are specifically intended for evaluating the unknown in terms of "less than" or "greater than" a definite set limit.

If it is desired that the specific heavy metal content be ascertained more closely, a series of knowns can conveniently be prepared in a set of tubes to be used alternatively in the B_1 position, these usually increasing from one tube to the next in lead content by 5 µg. Each of these must be matched in unit *B* with the unknown in unit *A*, as outlined above, before addition of the hydrogen sulfide T.S. Interpolation of values between the knowns yields results within the precision of the heavy metals determination by official methods.

In those instances necessitating the processing of the unknown in an alkaline solution the procedure has likewise been successful, 5 drops of a 10% aqueous solution of sodium sulfide being substituted for the hydrogen sulfide T.S.

Some chemical items requiring alkali to produce solution may be neutralized and then acidified with acetic acid without crystallizing or precipitating. Thus, the original directions may be followed therefrom.

EXPERIMENTAL

In order to establish the validity of this procedure, a number of dyes were selected to act as color back-

grounds. Each was first subjected to the conditions of the test in aqueous concentrations of 1–500,000 and 1–1,000,000. Comparisons were then made against their respective controls of like concentrations but with 10 ml. of distilled water replacing the 10 ml. of hydrogen sulfide T.S. Three of these dyes were found to be completely resistant to the reducing action of the hydrogen sulfide for a period of one hour and were consequently chosen for performance runs in the determination. The dyes F.D. & C. Yellow No. 5—Tartrazine, F.D. & C. Red No. 2—Amaranth and F.D. & C. Blue No. 1—Brilliant Blue approximate the primary colors.

When the above outlined method was applied, using each of the dyes in an aqueous concentration of 1–1,000,000, a standard of 5 μg of lead was definitely discernible, in all cases, from its respective color background. In fact, in the instances of the red and the blue shades, the color difference from unknown to standard seemed to be sharpened or enhanced over that witnessed for the general official heavy metals test. This latter phenomenon was interpreted as being due to the component colors (dye and metallic sulfide) having the effect of producing a new shade rather than just a one-color development as in the usual heavy metals estimation.

Using the same dye concentrations as mentioned immediately above, studies were made to ascertain whether color development, due to metallic sulfide formation, followed the same trend in both units. As directed under the method, each observation unit was processed to that point where the colors were equalized. A standard solution containing 10 μg of lead was added to both A_1 and B_1 tubes, the contents mixed, hydrogen sulfide T.S. added, again mixed, and allowed to stand during ten minutes reaction time. Nearly all such studies resulted in identical color matches in the two units. Occasionally a variation could be noted and these were usually interpolated to be of the order of 2 μg of lead. On repeating such an estimation, it was not always possible to show this variation and it was concluded that under the conditions of evaluation, no doubt this represented the limit of sensitivity to be expected especially by the naked eye. Higher lead concentrations were not attempted in this study, since unknowns of a comparable level would allow dilution, thus requiring less sample and consequently resulting in less color.

Assuming that the sensitivity of the test is in the order of that shown in the experiment described above, in an assay representing one Gm. of substance in the 25 ml. of unknown solution, it is feasible to determine as low a metal contamination as 5 p. p. m. as lead. This experience duplicates the performance of the official procedure, the latter being free from the complications of color in the solutions under examination.

DISCUSSION

Besides the general rules of colorimetry, certain precautions must naturally be observed in the procedure. The rubber support rings for the inserted or suspended tubes must be maintained in their originally adjusted positions to insure correct results. Occasionally an unknown is discovered to be of such a chemical nature as to produce a foreign color when brought in contact with hydrogen sulfide. Often this foreign color does not develop in the alkaline medium with sodium sulfide. As was indicated in reference to the dyes used for the experimental background, some colored solutions undergo reduction in presence of hydrogen sulfide with a resulting diminution of shade or the development of an entirely different color. In such an instance, reduction of the color by other chemical means prior to submission to test should be attempted. Alert observation by the chemist should make it possible to detect either of the above-mentioned difficulties.

Perhaps the most outstanding disadvantage of the proposed method is the size of sample required. This is of quite definite significance when analyzing materials from research origin. Many of the items which today require such examination are found to be extremely costly. To this end, the possibility of semimicro application should not be overlooked. In some laboratories such a method may appear disadvantageous in that it seemingly does not lend itself to instrumentation.

Limitations of the method, in addition to those of the official procedure, are:

1. Unknown solution must not possess a color intensity such that prevents interpretation of that formed by the metallic sulfides.
2. The medium resulting from dissolving the unknown must not be of such high concentration or specific nature as to inhibit the development of the colored sulfides.
3. As pointed out above, sample must not be chromatically reactable with the sulfide.

It is felt that in the hands of the experienced analyst the method serves a very definite purpose. In this laboratory it has been used with a great deal of success for the determination of heavy metals in such items as sulfonamides, antibiotics, vitamins, quaternary ammonium compounds, and in the control of a number of organic intermediates in the manufacturing processes. It is particularly adaptable for direct application to many of the official items such as Gelatin, U. S. P., and Dextrose, U. S. P., which now require ashing, semiashing or some other form of pretreatment. Other naturally colored items such as Pectin, N. F., and Whisky, N. F., present color difficulties in conducting the official heavy metals test which can be overcome by the proposed method. While the method described herein is potentially adaptable to practically all official items now specifically examined for heavy metals, it is recognized that a considerable amount of additional study on individual items is necessary before suggesting this as an official procedure but the method is recommended as an improvement over the present official heavy metals test in those instances where interfering colors must be dealt with.

SUMMARY

A method is offered for the direct determination of heavy metals in substances producing somewhat colored solutions.

The suggested procedure is intended primarily as a limit test but has been used successfully for quantitative estimation of heavy metals content.

A proposal is made for the investigation of the outlined method in application to official items producing interfering colors in solution, and especially those requiring special pretreatment.

The principles of the test can be expanded to a number of measurements in general colorimetry.

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Fluorescence of Powdered Vegetable Drugs with Particular Reference to Development of a System of Identification*

By CHARLES R. CHASE, JR.† and ROBERTSON PRATT

An introductory section includes a brief résumé of previous observations on the fluorescence of crude drugs and drug products exposed to ultraviolet radiation. A key for identification of powdered vegetable drugs based on their fluorescence under ultraviolet radiation is presented. Possible application of the method and its shortcomings are discussed.

FLUORESCENCE excited by visible light is reported to have been observed first in 1602 by Cascariola, a cobbler living in Bologna, Italy (1). According to DeMent the first demonstration of fluorescence excited by invisible rays was provided by Sir George Stokes about the middle of the last century. He observed that a tube containing a solution of quinine bisulfate appeared bluish when held in appropriate regions of the violet band of the visible spectrum and that the blue color became more intense as the tube was moved progressively toward the shorter wave lengths and even when it was moved some distance into the dark region beyond the visible violet rays.

Applications.—During the nearly one hundred years that have elapsed since this observation, much important information about fluorescence has been acquired and has been applied in the development of modern concepts in certain phases of physics and chemistry. Practical applications have been made in analytical chemistry and in industry. However, despite the fact that the first observation of fluorescence in ultraviolet radiations was made on a plant product that is an important drug, i.e., quinine, and despite the fact that several investigators have reported differences in fluorescence of several crude drugs or their preparations under ultraviolet radiations, pharmacognosists have made comparatively little use of the phenomenon. The only practical applications in common use appear to be in the detection of contamination by rodents (1*a*) and the well-known fluorescence test for distinguishing rhapontic rhubarb from the official U. S. P. species and the N. F. VIII identity test for *Hydrastis*.

Heimstädt (2) described a fluorescence microscope in 1912 and suggested its practical application in pharmacognosy for the detection of small amounts of ergot in meal. The following year Wasicky (3) in extending these observations directed attention to the high degree of sensitivity of the fluorescence method of detecting the presence of certain drugs or their constituents. He showed, for example, that quinine could be detected under the fluorescence microscope even when diluted $1:10^6$. He further applied the technique to the detection of dandelion in chicory, of rumex in gentian, and to differentiation of various fibers.

There seems to have been little impetus given to this line of investigation in crude drugs, however, until 1929 when Göllner (4) reported on the difference in behavior of three groups of drugs which he classified as responsive, indifferent, or phosphorescent in ultraviolet radiations. Following this observation a number of workers reported on the fluorescence of crude drugs (either powdered or granulated), of a number of galenical preparations, or of isolated and purified constituents of the drugs (5–14). Most of these, however, were comparatively limited studies, directed toward detection of adulterants in crude drugs (7–10), the separation of two or three related crude drugs (7, 12), a mere cataloging of the behavior of official preparations of different pharmacopœias under ultraviolet radiations (5, 6, 10) or of the behavior of extracted active principles (11).

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† Recipient of the Kilmer Prize for 1948.

Leupin and Steiner (13) observed that some drugs which exhibited a similar fluorescence might be made to fluoresce differently by treatment with appropriate reagents, and they suggested that this differential behavior might have valuable applications in the identification of crude drugs; but a search of the literature has failed to reveal any large-scale systematic attempts to work out a satisfactory system for identifying crude drugs by means of their behavior when exposed to ultraviolet radiation.

The work reported in this paper was undertaken to study the possibility of developing a system of identification of powdered vegetable drugs based on their fluorescence characteristics. It is conceivable that such a system might possess distinct advantages over, or might serve as a useful adjunct to, the present methods that depend upon microscopic examination of histological features and on microchemical tests, both of which are quite time-consuming. Saving of time and resolution of doubtful specimens might be expected to be among the advantages of a system of identification based on fluorescence. Such a system has been worked out tentatively and is the subject of the present report. Further experimentation is required to develop satisfactorily all of the details but it is believed that the work is sufficiently advanced and is of sufficient interest to warrant presentation in its present form. The only major piece of apparatus that is required, i.e., a suitable source of ultraviolet radiations and appropriate filter, is relatively inexpensive and is common in most laboratories equipped for physical measurements.

EXPERIMENTAL

Materials and Methods.—The fluorescence of 151 identified powdered drugs under ultraviolet light was studied. The source of radiation was a mercury arc provided with a Corning ultra-type filter which transmits the near ultraviolet (above 3100 Angstrom units) and absorbs the wave lengths in the visible portions of the spectrum. It may be assumed that the 3650 band of the mercury arc was primarily responsible for the fluorescence that was observed in this work.

Since it was soon found that direct observation of the dry powders was unsatisfactory, several methods of mounting were investigated. Finally, the following three methods were adopted.

Method A.—A small amount of the powdered drug was suspended in a solution of nitrocellulose in amylacetate[1] on a microscope slide. The solvent evaporated and the slide was ready for use in a few minutes. The mounting fluid does not fluoresce when exposed to the wave lengths that are transmitted by the Corning filter that was used. Slides prepared by this method may be kept for several months without apparent change of fluorescent characteristics.

Method B.—A small amount of the powdered drug was placed on a slide and treated with 1 *N* NaOH in methanol and permitted to dry for approximately thirty minutes. Then the mounting fluid consisting of nitrocellulose in amylacetate was added as in Method A and the slide was ready for use in a few minutes. The fluorescence characteristics of slides prepared in this way gradually change over a period of several weeks.

Method C.—A small amount of the powdered drug placed on a microscope slide was treated with 1 *N* NaOH in methanol and was observed under the ultraviolet radiations while still wet. Observations are made most easily in a dark room but satisfactory results can be obtained merely working in a shaded corner of the laboratory if the slides are placed on a black background and are shielded from direct visible wave lengths.

RESULTS

When mounted as in Method A, 51 of the drugs (33.7% of those studied) exhibited fluorescence. All colors of the spectrum were represented and there appeared to be some correlation between the part of the plant used and the color of fluorescence. Of the drugs that fluoresced red, 95.5% were flowers, fruits, or leafy portions of plants; of those that fluoresced yellow, 85.8% were underground portions of plants; and of those that were blue, 71.5% were seeds. It is worthy of comment that all powdered seeds that exhibited fluorescence in this method of mounting emitted a blue color. In some cases drugs that fluoresced did not appear as a uniform color throughout, but had a spotted appearance. The spots were almost always yellow or green. The fluorescent spots frequently appeared in drugs, the main fluorescence of which was some other color so that the spots appeared as yellow or green flecks against a colored background. In other cases they constituted the only fluorescence of the powdered drug so that they were seen against a dark gray or black background.

The drugs that exhibited fluorescence were reasonably well segregated into groups, but more than 60% of the drugs were in the large group that did not fluoresce at all. It was in an attempt to break down this nonfluorescing group that mounting Methods B and C which involve pretreatment with NaOH were developed. This search proved fruitful since 93.4% of the drugs that were studied were found to fluoresce in one or more of the methods and it was found that the fluorescence of a given drug was generally different in the different methods. This seems to indicate that the fluorescence of the drugs was due to a number of different compounds and that several such compounds probably occur in a single species.

The exact color of fluorescence of each drug in each method of mounting was listed using Ridgeway's "Color Standards and Nomenclature" (15) as a guide. The extremely large number of shades of each color listed in this work makes matching any sample with a definite shade in the charts a comparatively simple matter. Occasionally it was found that more than one drug fluoresced the same color. In such cases, once the color had been satisfactorily determined, it was necessary to estimate the intensity of fluorescence. Ultimately this should be worked

[1] This is sold as Testor's Model Airplane Cement.

out quantitatively with reliable instruments. For the present purpose, however, simple visual observation and grading upon an arbitrary scale of intensity ranging from 1 to 4 was adequate. By comparison of the color and intensity of fluorescence of a drug mounted in each of the three ways cited, it is generally possible to identify it quickly. In practical identification of an unknown, the powdered drug is first classified according to its color or lack of color in Method A; then it is further classified according to its color or lack of color in Methods B and C, and, if necessary, according to the estimated intensity of fluorescence in Method C. For convenience and for permanent reference in the present investigation a color rating based on Ridgeway's standards (*loc. cit.*) was assigned to the fluorescence of each drug when mounted in each of the three ways cited above. An individual with a normal sense of color, however, can satisfactorily separate the drugs into comparatively small groups and in many instances can make a final identification without recourse to such an extensive color guide. These observations have led to the development of the following key for identification.

Key for Identification of Powdered Vegetable Drugs on Basis of Their Fluorescence in Ultraviolet Radiations

I. Colored in Method A

A. Blue in Method A
1. Colored in Method C
(*a*) Colorless in Method B
(1) Blue in Method C
Almond meal
Delphinium
(*b*) Blue in Method B
(1) Blue in Method C
Corydalis
Gelsemium
(*c*) Blue-green in Method B
(1) Blue in Method C
Spigelia
Veratrum viride
(2) Blue-green in Method C
Senega
Black mustard
(3) Green in Method C
Yellow mustard
2. Colorless in Method C
(*a*) Colorless in Method B
Chondrus
(*b*) Blue in Method B
Linum
Squill
(*c*) Blue-green in Method B
Kava-Kava
(*d*) Green in Method B
Staphisagria
(*e*) Yellow-green in Method B
Ignatis
Nux Vomica
(*f*) Blue spots in Method B
Strophanthus

B. Purple in Method A
1. Blue in Method C
(*a*) Colorless in Method B
Clove
Myrica
Wild Cherry Bark
White Oak
(*b*) Purple in Method B
Ipecac
(*c*) Blue in Method B
Cardamom
White Pine
Sassafras
Sumbul
(*d*) Blue-green in Method B
Ipomoea
2. Not blue in Method C
(*a*) Colorless in Method B
(1) Colorless in Method C
Kamala
(2) Green in Method C
Viburnum prunifolium
(*b*) Purple in Method B
(1) Blue-green in Method C
Leptandra
(*c*) Blue in Method B
(1) Green in Method C
Aspidosperma
(*d*) Green in Method B
(1) Colorless in Method C
Pulsatilla
(2) Violet in Method C
Scutellaria
(3) Green in Method C
Cimicifuga

C. Green in Method A
1. Blue in Method C
(*a*) Green in Method B
Guaiac
(*b*) Yellow in Method B
Caulophyllum
2. Blue-green in Method C
(*a*) Blue in Method B
Coto
(*b*) Yellow-green in Method B
Asarum
3. Green in Method C
(*a*) Colorless in Method B
Haematoxylon
(*b*) Blue-green in Method B
Lupulinum

D. Spots and another color in Method A
1. Purple color in Method A
(*a*) Colorless in Method B
(1) Blue in Method C
White Oak
2. Red in Method A
(*a*) Colorless in Method B
(1) Colorless in Method C
Pilocarpus
(2) Purple in Method C
Castanea
(*b*) Blue spots in Method B
(1) Violet in Method C
Spearmint

E. Spots only in Method A
1. Colorless in Method B
(*a*) Colorless in Method C
Physostigma
(*b*) Blue-green in Method C
Granatum
(*c*) Green in Method C
Areca
Frangula
2. Blue in Method B

Key for Identification of Powdered Vegetable Drugs on Basis of Their Fluorescence in Ultraviolet Radiations

(*a*) Blue in Method C
Red cinchona
3. Green in Method B
(*a*) Purple in Method C
Scoparius
4. Yellow spots in Method B
(*a*) Blue in Method C
Ceylon cinnamon

F. Yellow in Method A
1. Yellow in Method B
(*a*) Blue in Method C
Berberis
Calumba
Hydrastis
Xanthoxylum
(*b*) Orange in Method C
Curcuma

G. Orange in Method A
1. Blue in Method C
(*a*) Yellow in Method B
Red Saunders
(*b*) Orange in Method B
Sanguinaria
2. Green in Method C
(*a*) Colorless in Method B
Rhubarb

H. Red in Method A
1. Colorless in Method C
(*a*) Colorless in Method B
Cataria
Lobelia
Pilocarpus
Pimenta
(*b*) Purple in Method B
Cubeb
2. Violet in Method C
(*a*) Colorless in Method B
Belladonna leaf
Hamamelis leaf
Peppermint
Senna
Stramonium
Uva Ursi
(*b*) Green in Method B
Digitalis
Matico
(*c*) Brown in Method B
Hyoscyamus
(*d*) Blue spots in Method B
Spearmint
3. Purple in Method C
(*a*) Colorless in Method B
Salvia
Castanea
4. Blue in Method C
(*a*) Colorless in Method B
Sabina
(*b*) Blue in Method B
Coccus
5. Green in Method C
(*a*) Colorless in Method B
Buchu
Chimaphila
Trifolium

II. Colorless in Method A

A. Colored in Method C
1. Blue in Method C
(*a*) Colorless in Method B
Anise
Belladonna root
Colchicum root
Colchicum seed
Conium
Convallaria
Coriander
Phytolacca
Stillingia
Viburnum opulus
(*b*) Blue in Method B
Hydrangea
Krameria
Piper
(*c*) Blue-green in Method B
Willow
(*d*) Green in Method B
Jalap
Licorice
(*e*) Purple in Method B
Grindelia
Nutmeg
2. Blue-green in Method C
(*a*) Colorless in Method B
Aconite
Apocynum
Cantharis
Capsicum
Cornus
Eucalyptus
Gossypium
Juglans
Mace
Matricaria
(*b*) Green in Method B
Calendula
3. Green in Method C
(*a*) Colorless in Method B
Bitter Orange Peel
Blue flag
Canella
Caraway
Chenopodium
Cocculus
Ergot
Eriodictyon
Saigon Cinnamon
Santonica
Sarsaparilla
Zingiber
(*b*) Blue in Method B
Aspidium
Sabadilla
(*c*) Blue-green in Method B
Gambir
Serpentaria
Taraxacum
(*d*) Green in Method B
Valeriana
(*e*) Yellow-green in Method B
Sambucus
4. Yellow in Method C
(*a*) Colorless in Method B
Cascara Sagrada
(*b*) Green in Method B
Foenugreek
(*c*) Yellow in Method B
Althaea

(*Continued on Page 328*)

Key for Identification of Powdered Vegetable Drugs on Basis of Their Fluorescence in Ultraviolet Radiations *(Continued from Page 327)*

B. Colorless in Method C
  1. Colorless in Method B
     - Arnica
     - Asclepias
     - Bryonia
     - Calamus
     - Colocynth
     - Elm
     - Euonymus
     - Eupatorium
     - Illicium
     - Sandalwood
  2. Blue-green in Method B
     - Agaricus
     - Foeniculum
  3. Green in Method B
     - Inula
     - Pyrethrum flowers
     - Thyme
  4. Yellow-green in Method B
     - Lappa
  5. Yellow in Method B
     - Gentian
     - Orris
     - Quassia

DISCUSSION

The study of fluorescence of powdered drugs as a means of effecting their identification appears to possess distinct possibilities of practical application, especially in the case of similar drugs that may be more or less difficult to distinguish. For example, Ceylon cinnamon can be quickly and reliably distinguished from other common varieties such as China and Saigon by virtue of the fluorescent spots that appear when it is mounted as in either Method A or B whereas the latter two varieties are nonfluorescent in Methods A and B. All three varieties fluoresce in method C but Ceylon cinnamon fluoresces blue whereas the China and Saigon cinnamons both appear green. The latter two may be separated, however, by the difference in intensity of fluorescence. In all cases that require use of estimates of intensity of fluorescence for identification comparison of the unknown should be made with a reference standard slide prepared with a sample of known identity.

Satisfactory segregation of the drugs into comparatively small groups, and in many instances final identification, can be made readily by means of the accompanying key. For the most accurate work, however, one should prepare a color code table in which a definite designation of the color of fluorescence of identified drugs in each method of mounting is recorded. Then if a guide such as Ridgeway (15) or Séguy (16) is used to evaluate the colors it should be necessary to resort to estimates of intensity only occasionally. We do not yet have great confidence in identifications that are based entirely on estimates of intensity, since in some of these cases the intensity of fluorescence is none too bright even under the most favorable circumstances. The fluorescence method may prove valuable, nonetheless, for identification even of those drugs which show duplication of color, since there are seldom more than four or five drugs in each color group even when only relatively crude separations are made. Thus, an unknown may be placed rapidly as one of a small group of drugs that can then be examined minutely by the conventional methods in a comparatively short time. This avoids the necessity of considering a large number of drugs microscopically. Frequently the drugs in such a group are so dissimilar in histological characteristics that only a cursory examination is required to identify them microscopically after the primary separation on the basis of fluorescence has been made. When mounted in the same way a large number of drugs may emit the same wave lengths under excitation by near ultraviolet radiations, but generally comparison of their fluorescence in the three methods of mounting (A, B, and C) permits differentiation. Further refinements of technique and use of different solvents should be expected to make possible indubitable identification of each of the several drugs that fall into a single group in the present key. For example, use of a diffraction grating might be expected to reveal subtle differences in the colors emitted by different drugs that appear to the unaided eye to emit the same color. Introduction of a fluorimeter to accurately measure quantitative differences in intensity of fluorescence might prove helpful.

There appear to be two principal difficulties that may be encountered in the fluorescence procedure for identifying crude drugs. The first is that which might occur in discriminating between colors, between blue-green and blue, for example. Obviously, if an error were made in the primary separation, the investigator would proceed through part of the key inapplicable to the particular drug under examination, and a satisfactory identification could not be achieved. However, this difficulty is no greater in this method than is that encountered in any dichotomous or "multiple-choice" key. An individual with a normal sense of color discrimination should have no trouble on this score.

The most serious defect in the present system appears to be its inapplicability to the ten drugs that do not fluoresce in any of the methods tested and to many of the leaves which fluoresce red in Method A, as might be expected because of their chlorophyll content, and which show very similar color changes in Methods B and C. Evidence from experiments now continuing in this laboratory indicates that this problem is amenable to satisfactory resolution by the use of appropriate reagents to intensify differentially or quench fluorescence or to induce qualitative differences in the emission. For example, in one series of experiments, Belladonna, Hyoscyamus, and Stramonium were chosen for study. Experiments were performed with the powdered drug and with aqueous and alcoholic extracts. Two satisfactory tests were finally obtained employing alcoholic extracts prepared by using powdered drug and 95% ethyl alcohol in the proportions of 0.5 Gm. to 10 ml. The suspension was shaken for ten or fifteen minutes and then set aside for twenty-four hours in a tightly stoppered container at room temperature. Then it

was filtered and made up to the original volume with alcohol and tested as outlined below.

The reagents used in the tests that followed were:

I. $AgNO_3$.....Saturated aqueous solution
II. NaOH.....0.1 *N* aqueous solution
III. $HgCl_2$.....5% aqueous solution

Test No. 1.

1. Add 1 drop of Reagent I to 2 ml. of extract and note color of fluorescence under U.V., then
2. Add 3 drops of Reagent II to extract containing I and note color of fluorescence under U.V.

Test No. 2

1. Add 2 drops of Reagent II to 2 ml. of extract and note color of fluorescence under U.V., then
2. Add 1 drop of Reagent III to extract containing II and note color of fluorescence under U.V., then
3. Add 1 more drop of III and note color of fluorescence under U.V., and then
4. Add 2 more drops of III and again note fluorescence as above.

The colors emitted at the various stages of treatment are shown in Table I.

TABLE I.—COLOR CHANGES IN FLUORESCENCE OF ALCOHOLIC EXTRACTS OF THREE POWDERED DRUGS AT DIFFERENT STAGES OF TESTS NOS. 1 AND 2 (SEE TEXT)

Step	Reagent	Belladonna	Hyoscyamus	Stramonium
		Test No. 1		
1	1 drop No. I	Purple	Red	Red (slightly darker than Hyoscyamus)
2	3 drops No. II	Blue	Green	Brown
		Test No. 2		
1	2 drops No. II	Blue	Red	Red
2	1 drop No. III	Purple	Brown	Red
3	1 drop No. III	Bright blue-green	Gray	Red
4	2 drops No. III	Blue	Gray-green	Red-violet

The order of adding the reagents and their concentrations are important, as are the time intervals between additions of reagents and observation of fluorescence. In general, the colors darken with time and eventually become indistinguishable. An interval of approximately one minute between steps has been found convenient and satisfactory. For best results it is desirable to run known identified samples along with the unknowns under test. Preliminary trials have shown that stock alcoholic extracts may be kept for several weeks as reference standards without deterioration of fluorescence characteristics. Stability tests to determine how long such solutions may be kept without deteriorating are in progress. If it is certain that the drug in question is one of these three, i.e., Belladonna, Hyoscyamus, or Stramonium, an individual familiar with the tests can confidently make an identification without use of the reference standards. Both Tests 1 and 2 should be used, however, to provide a double check.

The tests listed above were the best ones found, but several other reagents also resulted in changing the fluorescence of the alcoholic extracts. These reagents included KCNS, $KMnO_4$, $(NH_4)_2MoO_4$, $AuCl_3 \cdot 2H_2O$, and tannic acid. These tests are not satisfactory and are included here only to indicate the wide variety of compounds that may be used to induce changes in the wave lengths emitted when the extracts are exposed to wavelengths in the near ultraviolet. Although further work is required, the results recorded to date indicate that a reliable system of classification based on fluorescence can be developed for the precise identification of the relatively few drugs, the resolution of which is questionable when classified according to the tentative key given herewith.

Aqueous extracts were prepared in the same manner as the alcoholic extracts, except that the suspensions were heated for ten minutes on a water bath and then were permitted to stand overnight. Addition of a solution of potassium ferrocyanide resulted in a distinctive change in the color of fluorescence only in the case of Belladonna. Other reagents tested were ineffective.

The results obtained in these tests show the importance of studying the role that the solvent and method of preparing the sample may play in determining or influencing the color and intensity of fluorescence. The work of Zechner and Gstirner (5, 6) emphasized this. Leupin (8) and Leupin and Steiner (12) also commented on this subject. Goodwin and Kavanagh (17) studied the fluorescence of acetone and of *n*-butanol extracts of roots of 135 species of plants distributed in 69 families and reported that nearly all of them emitted a bluish or white color. None was reported as emitting predominantly red or orange and only one was reported as having a yellow tint, whereas in our investigation 85% of the drugs that appeared yellow or orange in Method A were underground structures, i.e., roots, rhizomes, or corms, and a number were found that were red or green. Linsbauer (18) using different techniques reported yellow fluorescence in roots of legumes. The importance of the solvent can be further illustrated by the following example. Cascara Sagrada appears yellow in Method C. Substitution of ethanol, ethylene glycol, propylene glycol, or glycerin for methanol causes no readily apparent change in quality or intensity of fluorescence. If water, ether, benzene, or isopropyl alcohol are used as solvents, however, the drug fluoresces green when exposed to the ultraviolet radiation; if acetone is used it appears blue, and blue-green if chloroform is used. Other factors that are known to influence fluorescence characteristics and which should be studied in connection with investigations aimed toward developing a system of identification are *p*H and temperature (19) and perhaps age of the sample (20). It has been reported (20) that the fluorescence

of various dried food products changes with time of storage.

Although a correlation was noted between color of fluorescence and part of the plant used in this work, no correlation was observed between fluorescence and taxonomic position of the plant. Another outcome of this line of investigation might be further elucidation of the nature of some of the more obscure chemical constituents of crude drugs.

SUMMARY

The fluorescence of 151 identified powdered drugs exposed to near ultraviolet radiations, predominantly the 3650 line from a mercury arc, was studied. Drugs were prepared in three different ways for examination, i.e. (*a*) mounted in a solution of nitrocellulose in amylacetate, (*b*) treated for 30 minutes with 1 *N* NaOH in methanol and then mounted as in (*a*), and (*c*) mounted in NaOH as in (*b*) and examined immediately. Observations made on drugs mounted in these three ways led to development of a key for identification of powdered vegetable drugs based on their fluorescent properties. The possible advantages of such a system of identification and the weaknesses of the present key are pointed out.

APPENDIX

It has already been stated (page 328) that experimental results emanating from different techniques indicated that a number of leafy drugs which exhibit similar responses in Methods A, B, and C might be successfully distinguished from each other by means of two simple tests applied to alcoholic extracts of

TABLE II.—SUPPLEMENTAL TESTS WITH ALCOHOLIC EXTRACTS INTENDED TO DISCRIMINATE BETWEEN DIFFERENT VARIETIES OF CERTAIN DRUGS

Drug	Untreated (Alc. Ext.)	Test No. 1		Test No. 2	
		Step No. 1 ($AgNO_3$)	Step No. 2 (NaOH)	Step No. 1 (NaOH)	Step No. 2 ($HgCl_2$)
		(4 drops)	(3 drops)	(6 drops)	
Benzoin, Siam	Blue	Greenish	Dark green	Dark blue	.....
Benzoin, Sumatra	Blue	Greenish	Light green	Light blue	.....
Buchu, Long	Purple	.....	.....	.....	.....
Buchu, Short	Blue	.....	.....	.....	.....
		(5 drops)	(2 drops)	(3 drops)	(2 drops)
Cinchona, Red	Bright blue	Bright blue	Blue	Blue	Purple
Cinchona, Yellow	Blue	Blue	Dark blue-green	Green	Brown-black
		(2 drops)	(1 drop)	(4 drops)	(1 drop)
Cinnamon, Ceylon	Gray-blue	Gray-blue	Gray	Gray	Violet
Cinnamon, China	Gray-blue	Gray-blue	Blue-green	Light gray	Blue
Cinnamon, Saigon	Gray-blue	Gray-blue	Gray	Blue-green	Brown
		(1 drop)	(1 drop)	(3 drops)	
Ipecac, Cartagena	Bright blue	Bright blue	Light blue	Bright green	Light blue
Ipecac, Rio	Bright blue	Bright blue	Dark blue	Dark green	Dark blue
		(3 drops)	(1 drop)	(3 drops)	
Licorice, Russian	No fluorescence	No fluorescence	Brown	Faint dark green	.....
Licorice, Spanish	No fluorescence	Faint green	Green	Bright green	.....
		(6 drops)	(1 drop)	(2 drops)	(2 drops)
Mustard, Black	Bright blue	Blue	Dark blue	Green	Green
Mustard, Yellow	Bright blue	Bright blue	Light blue	Bright yellow-green	Bright green
				(10 drops)	(6 drops)
Pepper, Black	Dull blue	.....	.....	Dull blue	Light blue-green
Pepper, White	Dull blue	.....	.....	Dark blue-green	Dark green
					(Lacto-phenol—2 drops)
		(4 drops)	(1 drop)	(7 drops)	
Plantago Seed, Blonde	Bright blue	Bright blue	Dark blue	Bright green	Blue
Plantago Seed, French	Bright blue	Bright blue	Dark violet	Bright green	Violet
		(1 drop)	(3 drops)	(2 drops)	(6 drops)
Senna, Alexandrian	Red	Red	Green	Red	Brown
Senna, Indian	Red	Red	Light green	Red	Green
		(1 drop)	(2 drops)	(10 drops)	(8 drops)
Xanthoxylum, Northern	Bright blue	Bright blue	Blue	Bright blue	Bright blue
Xanthoxylum, Southern	Bright violet	Purple	Purple	Dull blue-gray	Dark purple

TABLE III.—COLOR CHANGES IN FLUORESCENCE OF ALCOHOLIC EXTRACTS OF LEAFY DRUGS AT DIFFERENT STAGES OF TESTS NOS. 1 AND 2

Drug	Plain (Alc. Ext.)	Test No. 1		Test No. 2			
		Step No. 1 (1 Drop $AgNO_3$)	Step No. 2 (3 Drops NaOH)	Step No. 1 (2 Drops NaOH)	Step No. 2 (1 Drop $HgCl_2$)	Step No. 3 (1 More Drop $HgCl_2$)	Step No. 4 (2 More Drops $HgCl_2$)
Belladonna	Red	Purple	Blue	Blue	Purple	Bright blue-green	Blue
Buchu, Long	Purple	Dark purple	Dark blue	Purple	Purple	Purple	Gray
Cataria	Bright pink	Bright pink	Dull violet	Bright pink	Bright pink	Bright pink	Light gray
Chimaphila	Red-violet	Red	Brown-red	Red-purple	Red-purple	Red-purple	Red-purple
Digitalis	Red-violet	Violet	Dark purple	Violet	Violet	Purple	Purple
Eriodictyon	Blue	Dark blue	Black	Blue	Blue	Blue	Blue
Eucalyptus	Light brown	Brown	Dark brown	Brown	Brown	Brown	Brown
Hamamelis Leaf	Red	Dark red	Dark red	Red	Red	Red	Red
Hyoscyamus	Red	Red	Green	Red	Brown	Gray	Gray-green
Lobelia	Bright violet	Bright purple	Dull blue	Bright violet	Blue	Blue	Blue
Matico	Red	Dark red	Dark brown	Red	Red-brown	Brown	Brown
Peppermint	Red	Red	Dark red-purple	Orange-red	Orange-red	Orange-red	Orange-red
Pilocarpus	Tan	Brown	Green	Tan	Tan	Tan-gray	Gray
Sabina	Red-violet	Purple	Blue-green	Purple	Purple	Purple-gray	Gray
Salvia	Red-violet	Red-violet	Purple	Red-violet	Red-violet	Red-violet	Purple
Senna	Light brown	Light brown	Green	Light brown	Green	Green	Green
Spearmint	Red	Red	Brown-black	Red	Red-purple	Purple-brown	Dark gray
Stramonium	Red	Red	Brown	Red	Red	Red	Red-violet
Uva Ursi	Purple-brown	Purple-brown	Red-purple	Purple-brown	Purple-brown	Purple-brown	Purple-brown

drugs. An example using Belladonna, Hyoscyamus, and Stramonium was cited (page 329). The purpose of this appendix is to present further evidence to support that view. These confirmatory tests are offered as a means of clarifying the classification and identification of some of the crude drugs, determination of which was questionable in Methods A, B, and C. The results of the tests are presented in the form of an appendix because it is believed that their inclusion in the body of the text would impair its continuity and coherence.

The techniques that were used are described on page 329. These tests make it possible not only to distinguish many leafy drugs from each other but they possess the added advantage that with suitable modification (indicated in Table II) they may be used to discriminate between different varieties of a number of crude drugs, such as, for example, between the red and yellow varieties of Cinchona, between the French and blonde forms of Plantago Seed, between different varieties of Ipecac, of Senna, of Licorice, and of Mustard, to mention only a few. In some cases the response of the alcoholic extract alone or treated with only one of the reagents is so distinctive that it is unnecessary to resort to the use of the three reagents listed on page 329. In the case of Plantago Seed it was helpful to substitute lactophenol for $HgCl_2$ in Test No. 2. The results of these several tests are shown in Tables II and III.

Since sodium hydroxide is used in these tests attention should be directed to the marked influence that hydrogen-ion concentration exerts on the fluorescence of liquid preparations (cf. Radley, J. A., and Grant, J., "Fluorescence Analysis in Ultraviolet Light," ed. 3, D. Van Nostrand Company, New York, 1939, p. 424.

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Sample Size of Parenteral Solutions for Sterility Testing*

By LILA F. KNUDSEN†

Two questions have been asked in regard to sampling for sterility testing: "What size of representative sample should be taken?" and "How much should sample size vary with lot size?" Both of these questions can be answered by using some mathematical calculations together with a few assumptions. Recent developments in sampling theory have shown that sometimes tremendous variations in the quality of lots passed are due to chance alone involved in selecting the sample. It has been shown that it is possible to predict the variations and the frequency with which they will occur. The results of the mathematical calculations on a particular sample plan can be shown graphically by means of a curve called an "operating characteristic curve." Much has been written on sampling; however, Grant (1) and the Statistical Research Group (2) summarize sampling developments quite well.

ASSUMING that the bacteriological test is accurate and detects contamination with microorganisms in a container when present, the following "operating characteristic curves" show the probability of passing lots having varying incidence of contamination on the basis of testing a sample of a certain number of containers without finding contamination. For instance, if a sample of 10 containers is tested from the lot that is known to be 10 per cent defective, what are the chances of finding no contamination in the sample and therefore passing the lot? Of course, if a sample is tested from a lot having no contamination, the lot will always be accepted.

First, possibly, a definition of the term "operating characteristic curve" is in order. It is a curve plotted from mathematical calculations[1] showing the percentage of defects in, or quality of, the *lot* (not the sample) on the horizontal scale and the probability of accepting the lot on the vertical scale. All of the operating characteristic curves shown here are based on the testing of a specified number of containers (or units) from the lot and rejecting the lot if *one* container in the sample tested is found contaminated. In other words, the lot is accepted only if all containers are found to be free of contamination. From the operating characteristic (or *OC*) curve it is possible to see what chances are being taken of passing a lot containing a given percentage of defectives in the *lot*.

The curve on Fig. 1 labeled $N = 100$, $n = 10$ is the *OC* curve for the sampling specification: *From a lot of 100 containers take a sample of 10; if no container is found to be contaminated, pass the lot.* (The lot size is indicated by N, the sample size by n on all figures.) This curve shows that such a sampling plan would result in passing 1 lot out of every 3 lots having 10 per cent of the containers in the *lot* (not the sample) contaminated. The probability is thus 34 out of 100 that a 10 per cent defective *lot* will be accepted if decision on whether or not to accept the lot is based on testing 10 containers and finding no contamination. The chances of accepting a lot in which 20 per cent are defective (contaminated) are 1 in 10, and of accepting a 30 per cent defective lot are 2 in 100. One time in 200 even a 40 per cent defective lot will be accepted.

Figure 2 shows the *OC* curves for sample size of approximately 15 from lots of differing sizes. The sample sizes corresponding to given lot sizes are:

$N =$ 50	$n = 13$
$N =$ 100	$n = 14$
$N =$ 300	$n = 15$
$N =$ 500	$n = 15$
$N =$ 10,000	$n = 15$

By increasing the sample size from 10 to about 15 it can be seen that the probability of accepting a *lot* containing 10 per cent defectives is decreased from 34 out of 100 to 19 out of 100 and the probability of accepting a *lot* containing 20 per cent defectives is 3 out of 100.

Figure 3 shows the *OC* curves for samples of about 20. Here:

$N =$ 50	$n = 17$
$N =$ 100	$n = 18$
$N =$ 300	$n = 20$
$N =$ 500	$n = 20$
$N =$ 10,000	$n = 20$

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[1] The probability of acceptance is calculated for several distinct points on the curve by means of the formula

$$\text{Prob. of accept.} = \frac{(N-d)!(N-n)!}{(N-n-d)!N!}$$

where N = lot size, n = sample size, d = number of defects in the lot, and ! = factorial [e.g., $4! = 4(3)(2)(1) = 24$].

For instance for $N = 100$, $n = 10$, and $d = 5$

$$\text{Prod. of accept.} = \frac{95!90!}{85!100!} = 0.584$$

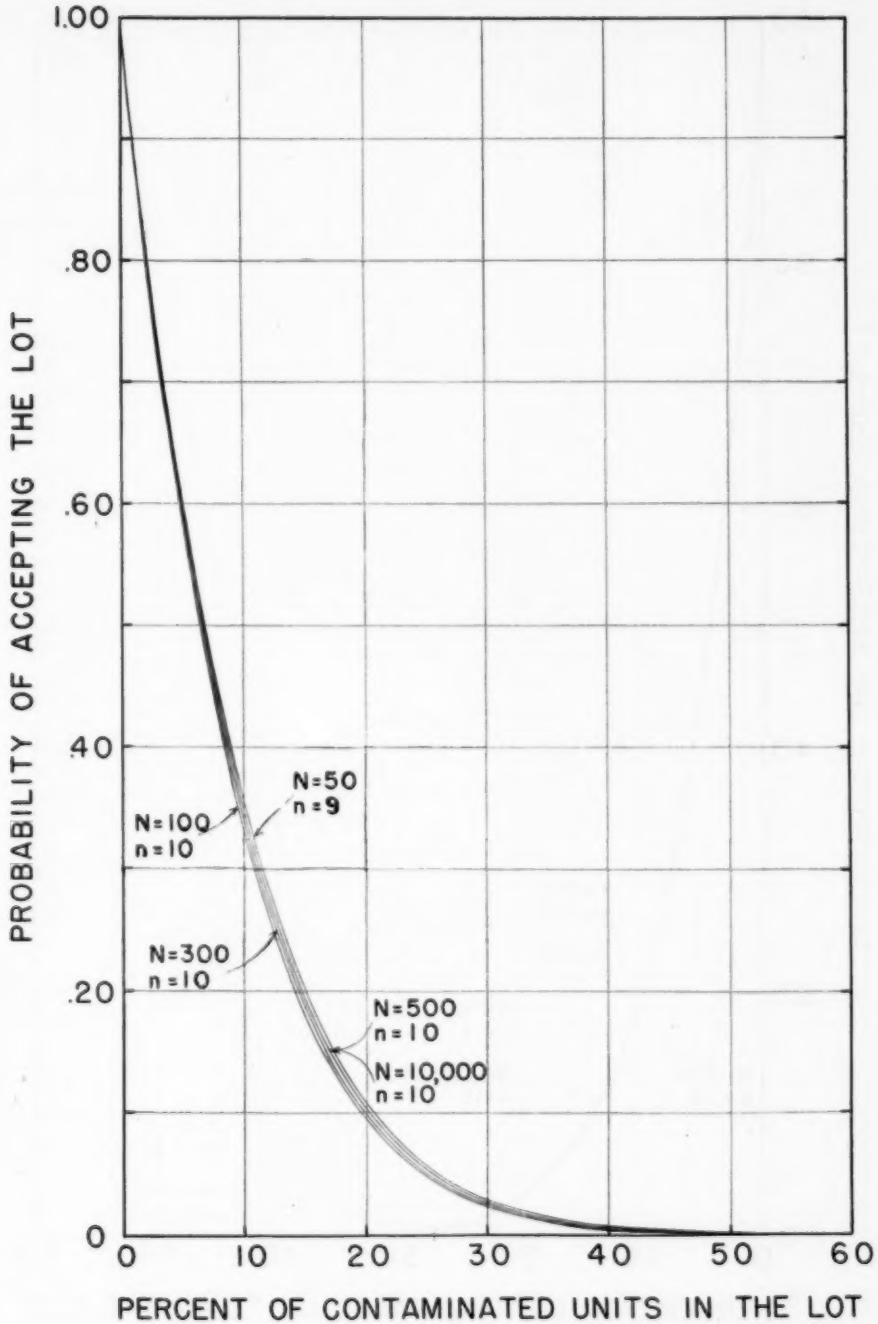

Fig. 1.—Operating characteristic curves for several lot sizes (N) and sample size of about 10 units. The lot is passed if there are no contaminated units in the sample tested.

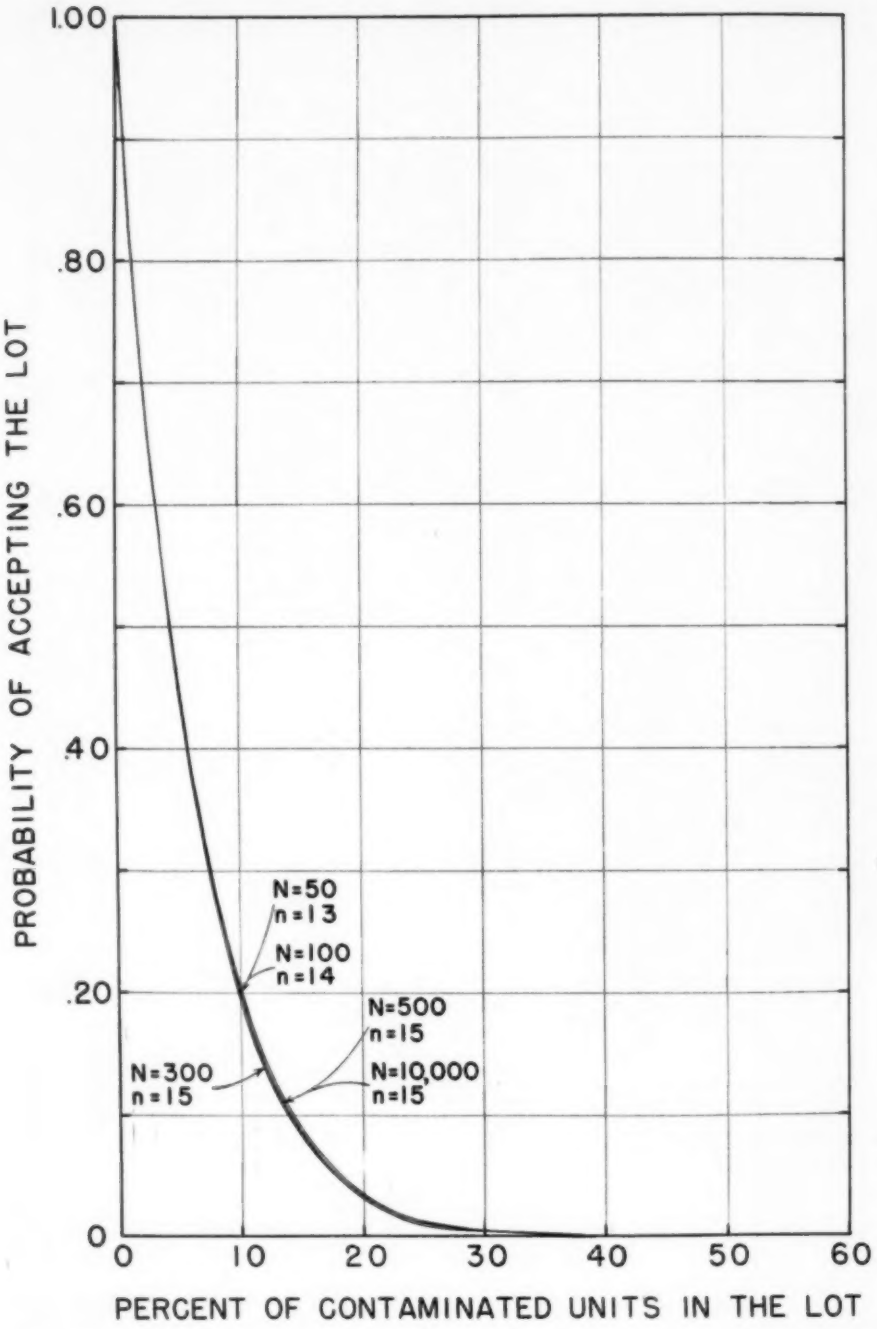

Fig. 2.—Operating characteristic curves for several lot sizes (N) and sample size of about 15 units. The lot is passed if there are no contaminated units in the sample tested.

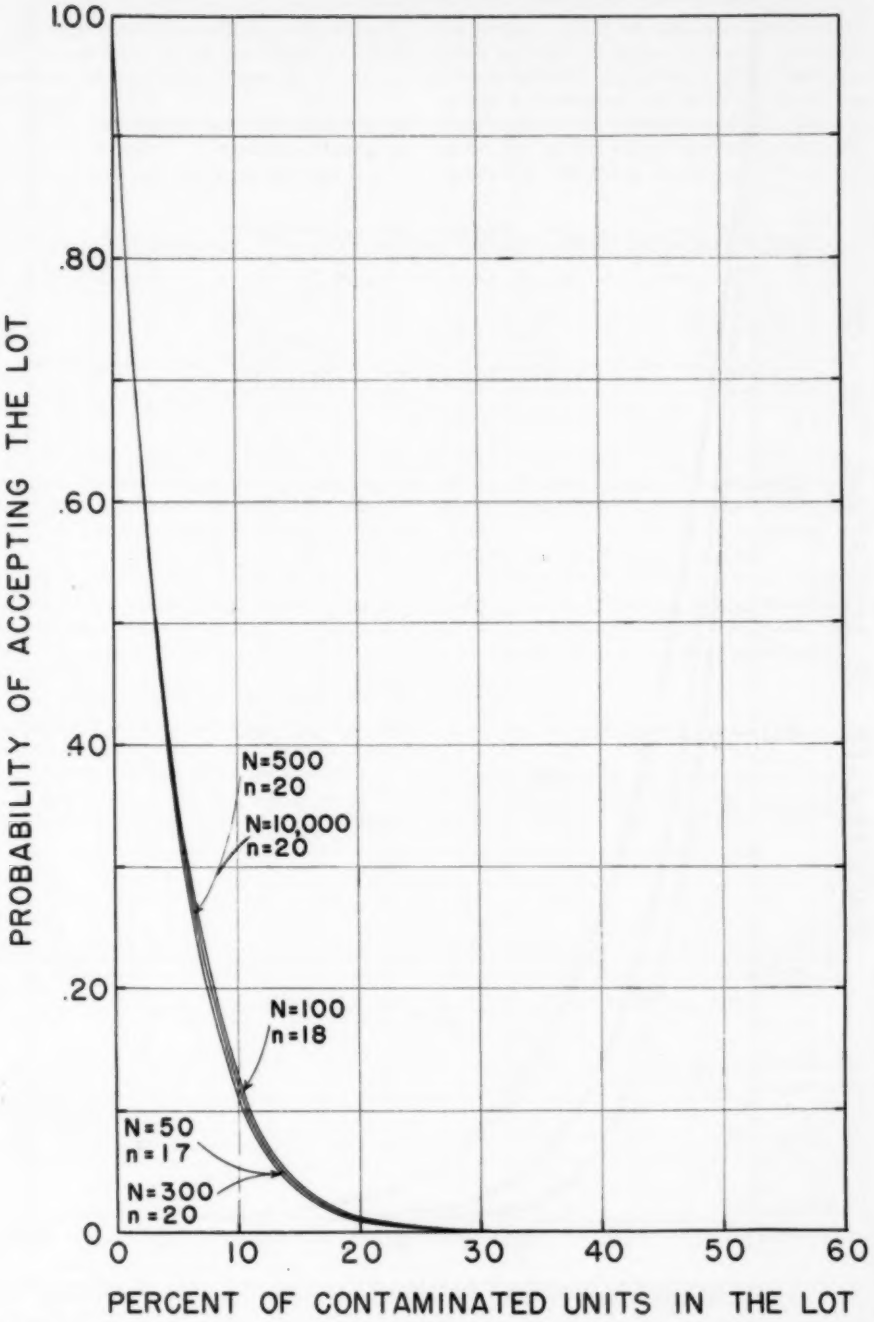

Fig. 3.—Operating characteristic curves for several lot sizes (N) and sample size of about 20 units. The lot is passed if there are no contaminated units in the sample tested.

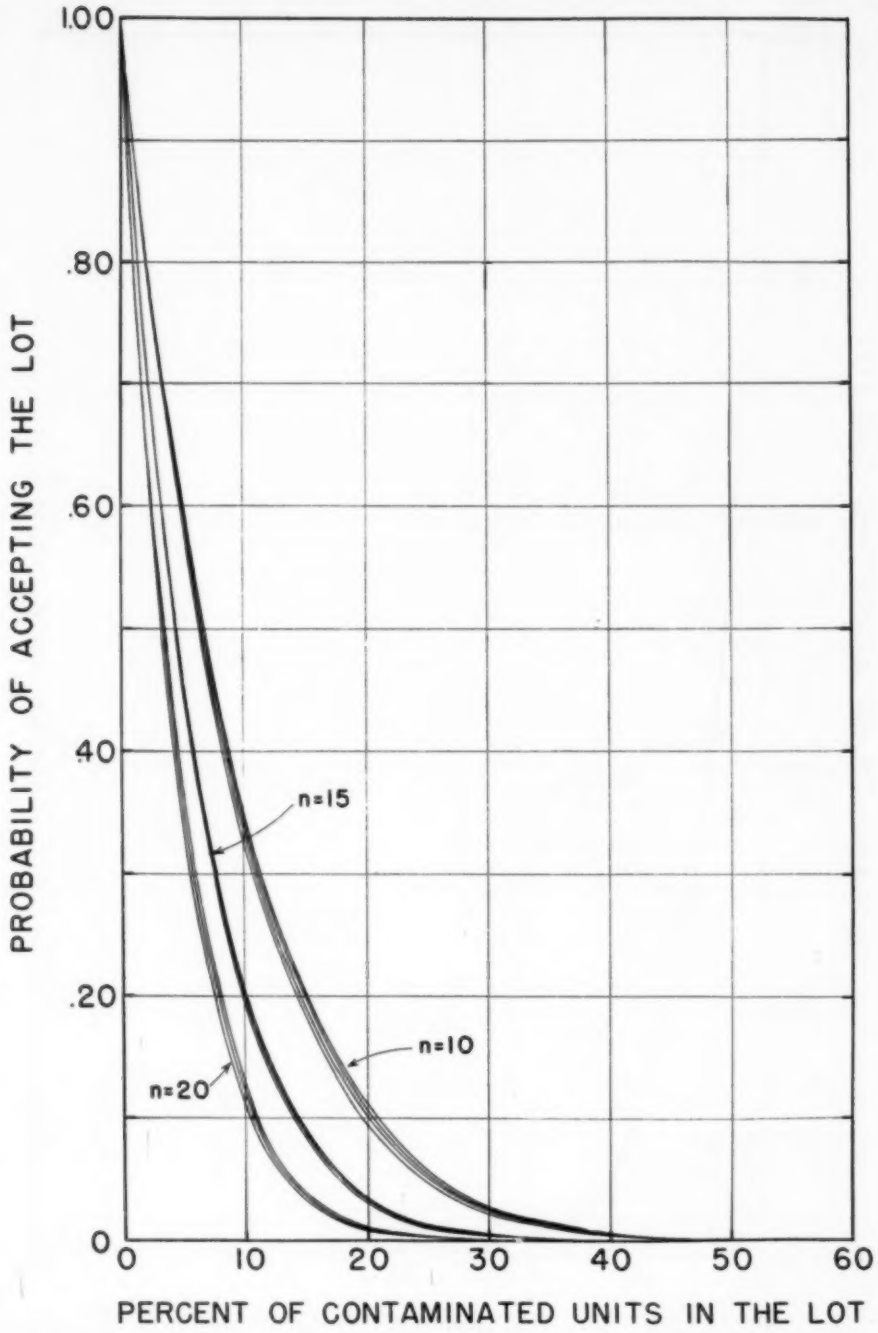

Fig. 4.—Operating characteristic curves for several lot sizes (N) and respective sample sizes (n). The lot is passed if there are no contaminated units in the sample tested. (Fig. 4 is a composite of Figs. 1, 2, and 3.)

The probability of accepting a *lot* containing 10 per cent defectives is still very large—namely, 1 in 9, and there is still one chance in 100 of passing a lot containing as much as 20 per cent defectives.

Figure 4 combines on one chart the *OC* curves for Figs. 1, 2, and 3. Comparative chances for different sample sizes are shown in Table I.

leniency with small lots than with large lots. For instance, given two lots, one large and one small, each with the same sample size, the respective probabilities of accepting a lot with, perhaps, 10 per cent defective will be the same. If the sample size of the smaller lot is decreased the probability of accepting a lot with, for example, 10 per cent defective is increased.

TABLE I.—CHANCES OF PASSING DEFECTIVE LOTS AS RELATED TO SAMPLE SIZE

% Defective in Lot (% of Contaminated Units in the Lot)	Chances of Passing Lot After Testing Sample of Size: $n = 10$	$n = 15$	$n = 20$
5	60 out of 100	45 out of 100	35 out of 100
10	34 out of 100	20 out of 100	11 out of 100
15	19 out of 100	8 out of 100	4 out of 100
20	10 out of 100	3 out of 100	1 out of 100
30	2 out of 100	5 out of 1000	1 out of 1000

Mathematical calculations were made to determine the size of sample to be taken from lots of 50, 100, 300, and 500 containers in order that the *OC* curve will most closely approximate that for $N = 10{,}000$ and $n = 10$. From these calculations it was found that the same size of sample ($n = 10$) should be taken for $N = 300$, 500, or 10,000 and that for $N = 50$, $n = 9$. As is obvious from Figs. 1, 2, and 3, the *OC* curves for approximately the same size sample are practically identical over a wide range of lot sizes. This means that the probability of accepting lots having a given percentage of contaminated containers is directly related to sample size rather than lot size.

If sample size increased directly or proportionately with lot size, it would result in greater

Thus decreasing the sample size with decreasing lot size would result in increasing the probability of passing lots having a given percentage defective. It would also result in passing lots having a greater percentage defective.

The inescapable conclusion is reached that since there is no reason to be more lenient with a small lot than with a large lot, the sample size taken must be approximately the same regardless of lot size.

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Analysis of Diisopropylfluorophosphate in Oil Solution*

By C. R. SZALKOWSKI, W. J. MADER, and H. A. FREDIANI

A combination of a total phosphorus determination with a so-called free hydrofluoric acid determination in the analysis of diisopropylfluorophosphate (DFP) in oil solution is described and discussed. The first part of this combination of determinations indicates initial DFP content while the second reveals the degree of hydrolysis.

ONE OF THE TOXIC materials investigated for wartime usage (1, 2) which has shown itself to be useful pharmacologically (3, 4) is diisopropylfluorophosphate (DFP). Because this chemical is strongly susceptible to hydrolysis, aqueous solutions have been found to vary in potency and to lose potency fairly rapidly (5). Since it is quite stable in oil solution, this drug has been introduced as a myotic and a 0.1 per cent concentration in peanut oil is sufficient to counteract a 4 per cent solution of homatropine bromide (3).

PROCEDURE

The problem involved in the adequate analysis of such oil solutions is extremely interesting from a

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chemical point of view. The hydrolysis proceeds according to the following equation:

$$2(C_3H_7O)_2POF + 2H_2O \rightarrow H_2F_2 + 2(C_3H_7O)_2POOH$$

resulting in the formation of an organic phosphate and hydrofluoric acid, neither of which apparently shows significant pharmacological efficacy.

Because of the low precision of animal tests, a chemical method for indicating the actual composition at a given time is highly desirable. Since degradation in oil solution proceeds slowly, precise chemical studies are required in order to evaluate properly the storage stability of such a product. A combination of a total phosphorus determination, to indicate initial DFP content of the preparation, together with a "free hydrofluoric acid" determination, to indicate degree of hydrolysis, has proved of value in our laboratory.

Determination of Initial DFP Content.—The total phosphorus present is converted to inorganic pyrophosphate by ignition in the presence of zinc oxide. Because of the small quantities involved, the colorimetric method for phosphorus originally reported by Eddy and De Eds (6) as modified for use in oils by Goodloe (7) has been found most satisfactory. Following the recommendations in the study by Kitson and Mellon (8), optical density measurements at 640 μ were used for which a photoelectric filter photometer with Corning filter No. 2404 served satisfactorily.

Reagents.—10 *N Sulfuric acid:* 282 ml. of reagent concentrated (sp. gr. 1.84) sulfuric acid diluted to 1 L. *Stannous Chloride:* Prepare a stock solution by dissolving 5 Gm. reagent $SnCl_2 \cdot 2H_2O$ in 25 ml. concentrated hydrochloric acid. For use, dilute exactly 1 ml. of stock solution to 100 ml. with distilled water. The stock solution is stable for at least two weeks; the dilute solution must be prepared fresh daily. *Molybdate Reagent:* Place 64 Gm. of reagent ammonium molybdate in a 1-L. flask; add 400 ml. distilled water and 500 ml. 10 *N* sulfuric acid. Dissolve by shaking and dilute to volume. *Phosphate Standard:* Dissolve 0.4392 Gm. reagent potassium dihydrogen phosphate in 500 ml. water and 5 ml. of 10 *N* sulfuric acid. Add 0.1 ml. of 0.1 *N* potassium permanganate (or sufficient to give a faint pink color) as preservative and dilute to 1 L. The resultant solution contains 0.1 mg. of phosphorus per ml.

EXPERIMENTAL

Accurately weigh 0.5 Gm. of sample into a small porcelain crucible and cover with 1 Gm. reagent zinc oxide. Heat gently over a low flame until the oil is all burned off, then ignite until free of carbon. Allow the crucible to cool and place in a 250-ml. beaker, removing the contents by tapping the crucible against the side of the beaker. Add about 40 ml. water and 10 ml. 10 *N* H_2SO_4. Heat on a hot plate with occasional stirring until solution is complete and then remove the crucible (rinse while removing). Transfer the solution to a 250-ml. flask and dilute to mark. Pipette a 50-ml. aliquot into a 100-ml. volumetric flask; add 0.2 ml. of *p*-nitrophenol indicator; and neutralize with 20% sodium carbonate solution (dropwise). Add 10 *N* H_2SO_4 dropwise until the solution clears and becomes colorless. Swirl after the addition of each drop. After neutralizing, add 10 ml. of the 10 *N* H_2SO_4 followed by 10 ml. of molybdate reagent. Rinse sides of flask until total volume is approximately 90 ml. and then mix thoroughly by swirling. Slowly add (while mixing) 5 ml. of the diluted stannous chloride solution, noting the time this is added. Dilute to 100 ml. and mix thoroughly. Transfer part of the solution to a colorimeter cell and, exactly ten minutes after addition of the stannous chloride, measure the transmission. Read the mg. of phosphorus directly from a calibration curve prepared with the instrument and reagents in use.

Calibration Curve.—Standard phosphate solutions are prepared by adding 0.005, 0.01, 0.02, 0.03, and 0.04 mg. of phosphorus to individual 100-ml. volumetric flasks, diluting to 50 ml. with water, adding 10 ml. 10 *N* H_2SO_4 and 10 ml. molybdate reagent, diluting to 90 ml., swirling, adding 5 ml. dilute stannous chloride, diluting to mark, and mixing. Transmission readings are taken exactly ten minutes after addition of the stannous chloride.

The colorimeter readings may be taken with the instrument set at zero with distilled water. A blank determination should be carried out with all of the reagents used and final colorimeter readings (for samples and calibration curve) should be corrected by subtracting the value of this blank. A new blank correction must be ascertained each time a new reagent is employed in the determination.

Calculation.—Total (initial) mg. DFP = mg. $P \times \frac{184}{31}$. In order to evaluate the efficacy of this procedure for phosphorus, known solutions of phosphate (as potassium dihydrogen phosphate) were added to the porcelain crucible together with peanut oil and put through the entire procedure. Recoveries of these known samples were 98.0, 98.6, 99.7, and 100.7% of theory.

Degree of Hydrolysis.—For the determination of the low fluoride ion concentrations involved, a modification of the ferric acetyl acetone photometric method of Wilcox (9) has been found to be most practical.

Since the test cannot be carried out in nonaqueous media a water extraction is necessary. By using multiple samples the necessary kinetic data may be obtained so that by extrapolating to zero time, the initial degree of hydrolysis may be calculated. A more thorough study of the kinetics of this hydrolysis will be published elsewhere.

Principle.—The hydrofluoric acid is extracted from the oil by shaking with water. The aqueous fluoride solution is then assayed by the colorimetric ferric acetyl acetone method.

Apparatus and Reagents:

Ferric Nitrate; dissolve 1.1 Gm. $Fe(NO_3)_3 \cdot 9H_2O$ in 500 ml. water.

Acetylacetone; dissolve 2.5 Gm. of freshly distilled acetylacetone in 500 ml. water.

Ferric acetylacetone; mix equal volumes of the ferric nitrate and acetylacetone solutions.

Standard fluoride solution; dissolve exactly 220 mg. reagent NaF and dilute to 1 L. 1 ml. = 0.1 mg. F.

Photoelectric cell colorimeter with 480 μ filter.

Colorimetric Ferric Acetyl Acetone Method.—Dissolve 5 ml. of sample in exactly 25 ml. absolute ether in a 125-ml. separatory funnel. Add (pipette)

25 ml. water and shake for thirty minutes. Allow the two layers to separate and draw off the clear, aqueous layer. If the aqueous layer is not perfectly clear (free of oil or ether), pass through a dry filter. To each of three 25-ml. volumetric flasks add exactly 2 ml. of ferric-acetylacetone solution. To one flask add a 10-ml. aliquot of the clear aqueous extract; to the second flask add 10 ml. of the extract, plus 1 ml. of fluoride standard (0.1 mg.); to the third flask, 10 ml. of water alone. Dilute to volume. Mix thoroughly and after ten minutes, read the color in a suitable colorimeter at 480 μ using distilled water for zero density. Calculate the results according to the following equation:

$$F = \frac{(B - S) \times 25 \times 0.1}{(S - X) \times 10 \times \text{weight of sample}}$$

where

F = mg. free fluoride per gram of sample,
B = optical density of reagents,
S = optical density of unknown,
X = optical density of unknown plus 0.1 mg. added fluoride.

The value obtained here is the free fluoride originally present due to hydrolysis of the sample plus free fluoride resulting from the thirty-minute contact with water. A hydrolysis time study has indicated this value to be within 15% of the original fluoride content of the sample. For routine control purposes, this value is sufficiently close to the truth.

To ascertain the efficacy of the fluoride analysis, together with that of the extraction procedure used, a standard solution of hydrofluoric acid in peanut oil was prepared, containing 0.82 mg. H_2F_2 per gram of oil. By the procedure detailed below recoveries of 97.0, 101.0, and 96.6% were effected.

DISCUSSION

A typical example of the data obtained for ascertaining initial hydrolysis is in order. A sample of diisopropylfluorophosphate in peanut oil was shaken with water at room temperature for periods of time varying from five minutes to five hours and the free fluoride determined as above. As may be expected, the free fluoride content increased with time as indicated in the following tabulation:

Time, Min.	Micrograms F
5	7.2
10	7.4
15	7.7
30	8.5
45	10.5
60	12.5
120	14.5
180	17.0
240	18.5
300	19.0

Because of the large excess of water present, it was presumed that this reaction would be a first-order reaction. A plot of log concentration of DFP, left at time t against t, yielded the typical straight line (Fig. 1) to be expected for a first-order reaction. Extrapolation of this line back to zero time indicates an initial hydrolysis of 7% for this particular sample. The reaction rate for this particular reaction is such that for routine testing of production samples a single determination, using a thirty-minute extraction time, suffices to yield a hydrolysis value within 15% of the initial. Analysis of five different lots for experimental evaluation resulted in the data tabulated below:

Sample	Labeled Conc. %, DFP	% DFP Found	% Hydrolyzed
A	0.1	0.100	20
B	0.05	0.047	12
C	0.1	0.093	8
D	0.1	0.101	7
E	0.1	0.100	3

The column labeled "% DFP Found" was obtained by calculation from the phosphorus determination. The "% Hydrolyzed" values are the maximum values obtained by calculation from the free fluoride concentration.

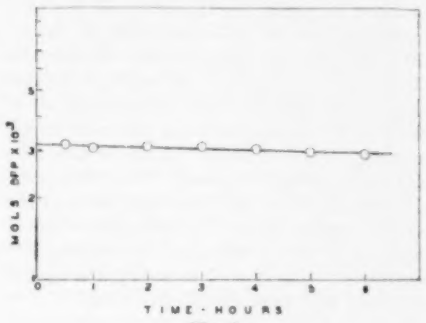

Fig. 1.

SUMMARY

1. A colorimetric phosphorus determination serves to indicate the initial diisopropylfluorophosphate concentration in oil solutions.

2. A colorimetric free fluoride determination indicates the hydrolyzed DFP content of such solutions.

3. The low moisture content of peanut-oil solutions of DFP decreases the rate of hydrolysis so as to result in relatively stable ophthalmic solutions.

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A Brief Historical Survey of Indian Drugs of Vegetable Origin*

By S. PRASAD†,‡

THE HISTORY of the medicinal use of plants in India can be traced to the remotest past. In the *Rigveda*, one of the oldest, if not the oldest, repository of human knowledge, and which is supposed to have come into existence about 4500 B.C., mention has been made of the "Soma" plant, which was given a place similar to the Greek *ambrosia*. In the *Atharva-veda*, which is a later production, the plants and vegetable products, in general, have been fully recognized as helpful agents in the treatment of diseases, though their use takes the form, in many instances, of charms and incantations. Thus the plant "Apamarg" (*Achyranthes aspera*), still considered in the Hindu system of medicine as a diuretic and laxative, is invoked as the "mistress of remedies" and "sole ruler over all plants" (19). It is in the *Ayurveda*, however, the foundation stone of the ancient medical science in India and which is supposed to have been compiled between 2500 and 600 B.C., that definite properties of drugs and their uses have been described in detail.

SCIENCE OF MEDICINE AND PHARMACOGNOSY IN ANCIENT INDIA

The two great works of this age (2500 B.C. to 600 B.C.) are the Charak and the Susrut. While in the Susrut (800 B.C. to 700 B.C.), surgery has been dealt with in great detail, the Charak (1000 B.C. to 800 B.C.) deals more with medicine. The simple medicines alone are arranged by this author under forty-five heads (Chopra, 1933). The methods of administration of drugs are fully described and bear a striking resemblance to those in use at the present time. About 2000 vegetable remedies have been included in the Charak, but only a few mineral drugs and still fewer animal remedies are cited (17). The soil, the season, and the gathering time of individual drugs of the vegetable kingdom are mentioned.

Ayurveda was a subject of study by all learned men, sages, and kings, for the benefit of the human race. The kings in particular encouraged the cultivation of medicinal plants as may be testified from the fact that Ashoka, the benevolent emperor (274–236 B.C.), established throughout his empire, hospitals for men and animals and botanical gardens for the culture of medicinal plants as sources of medicine for his people (16).

This was also the period of original research which resulted in remarkable progress in every respect. A number of books on *Ayurveda* and Hindu botany were written. Mention may be made of some of the important works by Vagbhatta, Madhavakar, Chakradatta, Srangdhar, Kanada, Sankarsen, and Bangasen (500 B.C. to 100 B.C.) who elaborated the vegetable *materia medica* of the Hindus and included many more new vegetable drugs. Bhoja-prabandha, a later work, contains a reference to inhalation of medicaments before operation and an anesthetic called "Sammohini" is supposed to have been used in the time of Buddha (4). The Hindu books on Botany, like "Kalpstanum" or "Vrikshayurveda," described detailed characteristics of vegetable drugs, their geographical distribution and habitat, soil suitable for their growth, the season for collecting them, the duration of their efficacy, and the methods of their storage. Elaborate classification of drugs and medicinal plants and detailed instructions on every conceivable point, such as the gathering time for different types of drugs, parts to be collected, manipulations and preparations, and the weights and measures to be used for dispensing, have been given.

HINDU MEDICINE AND ITS INFLUENCE ON FOREIGN COUNTRIES

From the time of Charak and Susrut up to about 1200 A.D., Hindu medicine made very good progress and attained its highest development. It made its way far beyond the shores of India—into Egypt, Greece, and Rome.[1] Dioscorides mentions in his work many Indian plants, particularly the aromatic group of drugs for which India has always been famous. Indeed Indian

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[1] The following quotation from "Culture and Commerce of Cotton in India," by J. Forbes Royle, p. 387 (Smith Elder & Co., 65, Cornhill, London, 1851) offers another aspect of the same fact: "If an indigofera had not been indigenous, indigo would never have derived its name from India, nor have afforded us the proof, in the stripe of mummy-cloth, of the early commercial intercourse between its native country and Egypt. Neither would sugar have been arranged by the Greeks with honeys, . . ."

perfumes, unguents, and similar articles of luxury were then in great demand in Rome and other countries. For instance, Ktesias of Knidos, who was physician to Artaxerxes Mnemon about 400 B.C., refers to cinnamon oil exported from India (26). The anonymous author of *Periplus* states that the export from Sind included *Costus* (*Saussurea lappa*). Pliny also refers to a heavy drain of Roman gold to India in buying costly drugs and aromatic spices (Pliny, "Historia Naturalis," Vol. XXIV, p. 1).

Toward the close of this period, Charak's fame traveled into Arabia. In the eighth or ninth century A.D., Charak's work was translated and was well known in Arabia. Rhazes (865 to 925 A.D.) calls him Scarak, Avicenna (980 to 1035 A.D.) quotes him as Scirak, and Serapion mentions Charak by the name as Zarch. The fact that several standard Hindu works on medicine and *materia medica* were translated into Arabic (Dietz, "Analecta medica"; Wustenfeld, "Geschichte der Arabischem Aerzte" (1840); Fluegel and others), and that several Indian drugs like pepper, lac, nard, licorice, asafetida, *Ocimum sanctum*, bdellium, cinnamon, myrrh, red sandal, Calamus (*Acorus calamus*), and the chebulic myrobalans have been included in the *materia medica* of Useibiah and others, indicates how much Arabic medicine was influenced by the Hindu medicine (Ray, 1902).

POSITION OF HINDU MEDICINE IN MEDIEVAL INDIA

The vegetable drugs employed by the Hindu physician and contained in the various books extant at the period mentioned above ran into several thousands, but during the invasion of India by the Greeks, Scythians, Huns, and the Mohammedans successively, a good deal of the existing Ayurvedic literature was mutilated or lost. Already the Buddhistic doctrine of *Ahimsa* (Non-killing), which led people to consider touching and dissection of dead bodies as sinful, had resulted in a decline of surgery. Medicine, however, had maintained its progress during the Buddhistic period and a large number of vegetable drugs were added to the already extensive *materia medica*. By the eleventh century or so, decadence set in generally, in knowledge, learning, and practice of both medicine and surgery.

When the Mohammedan rule was established in India from the thirteenth century on, the Arabian or the medieval Greco-Arab system, also known as the "Unani-Tibb," became the state system of medication. This system was also fairly advanced for that period, so that the old Hindu system of treatment was pushed to the background. The Arabic system brought with it a rich store of its own *materia medica*, unknown to India. During the long period of Mohammedan rule (up to the seventeenth century), this system came in close contact with the Ayurvedic or the Hindu system of medicine, resulting in a great deal of intermingling.

DECLINE AND EVOLUTION OF THE INDIGENOUS DRUGS OF INDIA

Both the Ayurvedic and the Unani-Tibb systems gradually declined with the fall of the Moghul Empire, coinciding with the advent of the Europeans in India—first the Portuguese, then the French and last the English. When the British had established their rule, the Western system of treatment was introduced in India. This further threw the indigenous systems (Ayurvedic and Unani-Tibb) into the background; nonetheless, both the ancient systems have remained the main source of medical relief to the bulk of the Indian population up to the present.

The modern Western system brought with it its own *materia medica*. Many of the indigenous drugs, however, after some pharmacological, chemical, and clinical investigations, were included in the British Pharmacopœia, and some of these like Kurchi, Ispaghula, Chirata, and Kaladana were accepted by the B. P. 1914. In the "Indian Pharmacopœial List" which has been published under the authority of the Government of India, all these drugs have been given recognition and many more like Rauwolfia, Punarnava, Vasak, etc., have been included. This "List" is considered of the same authority as the supplements to the B. P. published in Canada, Australia, and other dominions.

The earliest attempts at reviving the Indian drugs were confined mainly to the collection of available information about various medicinal plants. Sir William Jones' "Botanical Observations on Select Indian Plants" was one of the earliest contributions in this field (13). This was followed by John Fleming's "Catalogue of Medicinal Plants" (12), Ainslie's "Materia Medica of the Hindustan" (1), Roxburgh's "Flora Indica" (20), and later the works of Wallich (29), Royle (21, 23), and others (Strachey, 1852; Boissier, 1867; Stewart, 1874; Kurz, 1877; and Hooker 1875–97) (28, 2, 27, 15, 10). These did much toward resolving some degree of scientific arrangement from the chaos which existed in the vast mass of India's botanical material.

Of the earlier work on *materia medica* and the pharmacological aspect of Indian drug plants, mention may be made of O'Shaughnessy's "Bengal Pharmacopœia" (18), Irvine's "Materia

Medica of Patna" (11), "Pharmacopœia of India" by Dr. Waring (30), Dr. Mohideen Sheriff's "Supplement to the Pharmacopœia of India" (25), "Pharmacographia" by Flueckiger and Hanbury (9), Dutta's "Materia Medica of the Hindus" (6), and Dymock's "Vegetable Materia Medica of Western India" (7). But the two comprehensive works, namely, "Pharmacographia Indica" by Dymock, Warden, and Hooper (8) and "Dictionary of Economic Products of India" by Sir George Watt (31) are by far the most valuable of all the previous works and remain, with few exceptions, even today the final source for scientific reference. Works published later such as Kanny Lal Dey's "Indigenous Drugs of India" (5) and Kirtikar and Basu's "Indian Medicinal Plants" (14) are largely compilations from the last-named literature.

"Pharmacographia" by Flueckiger and Hanbury deals with vegetable drugs in England and in British India, while "Pharmacographia Indica" by Dymock, Warden, and Hooper is the pioneer work on the chemical aspect of Indian vegetable drugs. The Indigenous Drugs Committee (1901–1916) also did useful work in this line.

A perusal of "Indian Medicinal Plants" by Kirtikar and Basu, which is in four volumes and is provided with well-illustrated plates, testifies to the fact that a very large number of medicinal plants employed in India grow in different parts of the country. Indeed it has been said that about 2500 plants having alleged medicinal properties flourish there; these include about 300 plants mentioned in the British Pharmacopœia and the British Pharmaceutical Codex (3). Moreover, due to varied climatic and soil conditions India has the potentiality of producing exotic drugs, ranging from those growing in the hottest, tropical, and damp climate to those thriving in the dry, temperate, and cold regions. Cinchona, ipecac, digitalis, and pyrethrum may be taken as examples. However, as revealed by Chopra and co-workers (4), not many of the numerous indigenous drugs can be said to have any marked physiological activity. Much professionalism and orthodox belief in the curative properties of the indigenous drugs exist. A long and thorough scientific investigation is essential for sifting out the potent drugs from those devoid of any marked activity.

Of late years, a spirit of inquiry and research into the ancient systems has been manifested among the people. Detailed chemical and pharmacological investigations have been undertaken by some of the research institutes and medical colleges, notably the School of Tropical Medicine, Calcutta; the Haffkine Institute, Bombay; the Research Institute, Medical College, Madras; and the University of Travancore Research Institute. No doubt, with careful pharmacological, chemical, and pharmacognostical studies, claims concerning quite a number of the ancient Indian drugs will be justified.

There is a large group of people in India who still favor the revival of their indigenous systems of medicine. They argue that apart from economic considerations, these drugs are suited to the habits of the people and the climatic conditions that prevail in the country. In spite of a very strong reaction and inclination in this direction, there seems to be a clear understanding that medicine is universal. Hence it is to be hoped that before long the evolution now going on in India will lead to results useful to the Western as well as to the Indian people.

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Derivatives of Benzoic Acid and Simple Phenols in the Chemotherapy of Tuberculosis*,†

By F. A. FRENCH and B. L. FREEDLANDER

Bacteriostatic values of certain selected simple phenols, derivatives of benzoic acid and of salicylic acid, coumarins, and benzophenones against *M. tuberculosis* have been determined and the results are reported. Certain of these agents were also tested against other microorganisms, and *in vivo* in guinea pigs infected with *M. tuberculosis*, using streptomycin and *p*-aminosalicylic acid as reference drugs. A theory of the mechanism of action of salicylic acid and some of its derivatives is proposed.

THE GENERAL interest in aromatic acids as possible tuberculostatic chemotherapeutic agents rests on certain primary findings. Saz and Bernheim (3, 6), Bernheim (5, 9), and Fitzgerald and Bernheim (10) found that the oxygen uptake of *M. tuberculosis* strains H37Rv and B was stimulated greatly by salicylic acid while *M. tuberculosis* strain No. 607 was unaffected. The effect was apparently catalytic and the compound was not grossly metabolized. They found the meta and para isomers were not stimulatory to the pathogenic strains; however, recent investigations disclosed that all three compounds (Table I, Nos. 2, 3, 4) were mildly bacteriostatic. Fitzgerald and Bernheim (10) also found that cultures of *M. tuberculosis* H37Rv, adapted to growth in a salicylate concentration of 25 mg. per cent, were changed microscopically, culturally, and manifested increased virulence. In addition the adapted strain was less sensitive to *p*-aminosalicylic acid (PAS) inhibition.

These observations are of great interest and fundamental importance. Structural modifications of salicylic acid have led to the development of one agent, PAS (Lehman 12), of value in experimental tuberculosis and of definite clinical interest. In addition the present work and the work of Saz and Bernheim and co-workers (3, 7, 11) have shown that many compounds related to salicylic acid are highly bacteriostatic and highly specific for virulent tubercle bacilli.

In the following experiments a group of simple, related benzoic acid and phenol derivatives have been evaluated for tuberculostatic action *in vitro*. Additional *in vitro* studies were conducted with *M. leprae*, *M. phlei*, *M. tuberculosis* No. 607, *Trichophyton gypseum*, and a monilia (*Candida crusei*). Several of the compounds studied were investigated for their *in vivo* therapeutic action in experimentally induced tuberculosis in guinea pigs. Some of the compounds and a number of related compounds have been studied by other investigators (1–12). In the following work an attempt is made to correlate the results obtained here with those of other authors.

METHODS

The bacteriostatic studies were performed on virulent human tubercle bacilli strain No. H37Rv in Proskauer and Beck synthetic glycerin and asparagin media at a *p*H of 7.5. The chemicals were dissolved with a minimum of heating in propylene glycol or in 0.8% sodium hydroxide solution to make 0.5% solutions. Subsequent dilutions were made directly into the media without further heating. Each tube was inoculated with one loopful (4 mm. in diameter) of a fourteen-day-old surface culture of tubercle bacilli. These surface cultures were read at eighteen days using the highest dilution in which there was less than 50% of control growth as an end point.

Some additional tests were conducted with a few selected chemicals on *M. tuberculosis* No. 607 (a saprophytic mycobacterium), *M. phlei*, *M. leprae*, a monilia (*Candida crusei*), and *Trichophyton gypseum*. The methods of making dilutions, of inoculation, and of reading bacteriostatic end points were substantially the same as described for *M. tuberculosis* H37Rv. *M. leprae*, *M. phlei*, and *M. tuberculosis*, No. 607 were grown in Proskauer and Beck media at a *p*H of 7.2. They were incubated at 37° for five days, three days, and three days, respectively. *Trichophyton gypseum* was grown on Sabouraud's dextrose agar at a *p*H of 5.6 for five days at 22°; *Candida crusei* was grown on the same media at 37° for two days. In the case of the solid media the proper dilutions were made at 60° and the tubes were then slanted with no subsequent heating.

Animal Experiments.—Twelve guinea pigs weighing an average of 400 Gm. were used to evaluate each compound. The animals were inoculated in the left groin with 1.0 mg. wet weight of H37Rv tubercle bacilli. The drugs were administered once daily, orally by pipette, in aqueous suspension buffered to neutrality by sodium bicarbonate. Therapy was started on the day of infection. The dosage of all drugs was increased, gradually, for the first two weeks to the approximate chronic maximum tolerated dose; thereafter the dosage was reduced by 20%. The experiments were terminated at the end of six weeks. Pathology was evaluated numerically, based on a maximum of four for each organ (glands, spleen, liver, and lungs). Hemoglobin

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and weight determinations were made at the end of the experiments.

RESULTS

The *in vitro* bacteriostatic values are recorded in Table I. These values are selected from several experiments and may be considered to have a probable accuracy of ±1 dilution under the test conditions. The most extensively investigated compounds were the 4-ethers of 4-hydroxy and 2,4-dihydroxy benzoic acids. A gradual rise in bacteriostatic activity with increasing molecular weight of the ether group was noted in both series and the values were highest in the amyl ethers (compounds 13, 14, and 20). The simple phenols (compounds 26–30, inclusive) were mildly inhibitory. The compounds were bacteriostatic rather than bactericidal. Compounds 12, 19, 21, and 25 were tested against *M. leprae* and found inactive at a concentration of 10 mg. % while compound 30 just inhibited at 10 mg. %. Compound 15 was inactive against *M. phlei* at 0 mg. %. The monilia and *Trichophyton* tested were unaffected by 10 mg. % concentrations of compounds 19, 22, and 30. Compound 12 inhibited the *Trichophyton at 2.5 mg. %.*

The *in vivo* results are summarized in Table II. With the possible exception of compounds 33 and 35 which are similar structurally and gave low positive results, all compounds tested, other than the reference drugs (compounds 36 and 37), were devoid of *in vivo* activity. The value of these results consequently rests only in their correlative and extrapolative significance.

DISCUSSION

Our first interest in these simple aromatic compounds as tuberculostatic agents stemmed from consideration of the observation by Anderson and Newman (13) that *p*-anisic acid is a constituent of the lipoid fraction of virulent tubercle bacilli. We noted that it was not bacteriostatic and seemed to stimulate growth somewhat. The possible significance of this compound in the metabolism of tubercle bacilli appears to have been completely ignored. Following theoretical considerations closely analogous to those of Buu-Hoi (14), we undertook the synthesis and testing of a number of compounds bearing close structural relationships to *p*-anisic acid. When the methyl group was replaced by higher alkyl groups (compounds 10–13), inhibitors resulted. Similarly the *p*-alkyl ethers of 2,4-dihydroxybenzoic acid inhibited at almost exactly the same concentrations. These derivatives of 2,4-dihydroxybenzoic acid may be regarded as close polar analogs of PAS but their level of inhibition and quite possibly their mechanism of action is very different. It is interesting to note that additional nuclear substitution gave decreased bacteriostatic activity.

Umbelliferone and β-methylumbelliferone were included because of their vinylogous relation to 2,4-dihydroxybenzoic acid. Compound 33 can be regarded as a vinylog of salicylic acid (No. 2); compound 34 as a vinylog of *p*-hydroxybenzoic acid (No. 4); and compound 35 as a vinylog of 3,5-dichlorobenzoic acid. These benzophenones (compounds 33–35) possess the same type of hydrogen bond ring structure found in salicylic acid. The fact that activities are maintained or even increased in the vinylogs is encouraging and points to certain possible further extensions in this group of drugs. The principle of vinylogy may apply in these cases because the critical spacings between polar centers are not grossly changed.

No adequate correlative interpretation of the antituberculous activity of simple salicylic acid

TABLE I.—BACTERIOSTATIC ACTION ON MYCOBACTERIUM TUBERCULOSIS STRAINS H37Rv AND 607[a]

No.	Compound	Min. Inhib. Conc. Mg. %[b] H37Rv	607
1	Benzoic Acid	5.	...
2	2-Hydroxybenzoic Acid	2.5	50
3	3-Hydroxybenzoic Acid	5.	50
4	4-Hydroxybenzoic Acid	2.5	50
5	2-Methoxybenzoic Acid	10.	50
6	3-Methoxybenzoic Acid	10.	>50
7	4-Methoxybenzoic Acid	>10.	>100
8	4-Methoxy-3-aminobenzoic Acid	10.	100
9	4-Methoxy-3,5-dichlorobenzoic Acid	>10.	50
10	4-Ethoxybenzoic Acid	2.5	100
11	4-*n*-Propoxybenzoic Acid	1.	...
12	4-*n*-Butoxybenzoic Acid	0.25	...
13	4-*n*-Amoxybenzoic Acid	0.025	50
14	3-*n*-Amoxybenzoic Acid	0.025	...
15	4-Methoxy-2-hydroxybenzoic Acid	2.5	...
16	4-Ethoxy-2-hydroxybenzoic Acid	2.5	>10
17	4-*n*-Propoxy-2-hydroxybenzoic Acid	1.	...
18	4-Isopropoxy-2-hydroxybenzoic Acid	1.	...
19	4-*n*-Butoxy-2-hydroxybenzoic Acid	0.25	50
20	4-*n*-Amoxy-2-hydroxybenzoic Acid	0.025	...
21	2,4-Dihydroxybenzoic Acid	1.	...
22	2,4-Diacetoxybenzoic Acid	1.	...
23	2,4-Dihydroxy-5-chlorobenzoic Acid	>10.	...
24	4-Ethoxy-5-chloro-2-hydroxybenzoic Acid	2.5	...
25	Orsellinic Acid	10.	...
26	Phenol	5.	...
27	Catechol	2.5	...
28	Resorcinol	10.	...
29	Hydroquinone	5.	...
30	Orcinol	1.	10
31	Umbelliferone	1.	...
32	β-Methylumbelliferone	0.25	...
33	2,2′-Dihydroxybenzophenone	1.	...
34	2,4′-Dihydroxybenzophenone	1.	...
35	2-Hydroxy-3,5-dichlorobenzophenone	0.25	...
36	*p*-Aminosalicylic Acid[c]	0.0025	...
37	Streptomycin[c]	0.001	...

[a] The compounds in this table were prepared in this laboratory under the direction of F. A. French with the following exceptions: Nos. 1–7 and 26–30, inclusive, were obtained from the Eastman Kodak Co. (highest purity); Nos. 21 and 32 were obtained from The Koppers Co.; No. 35 was generously supplied by the Dow Chemical Co.; No. 36 by the Parke, Davis & Co.; and No. 37 by E. R. Squibb and Sons.

[b] These are the minimum concentrations giving greater than 50% inhibition of bacterial growth.

[c] *p*-Aminosalicylic acid and streptomycin were included for purposes of comparison.

TABLE II.—SUMMARY OF *in Vivo* EXPERIMENTAL DATA

No.	Compound	Av. Daily Dosage, Mg. per Animal	Path. Ratio[a]	Relative Wt. Gain, Gm. Deviation from Controls	Hemoglobin % Deviation from Controls
8	4-Methoxy-3-aminobenzoic Acid	90	1.16	−6	0
9	4-Methoxy-3,5-dichlorobenzoic Acid	151	1.02	−35	+2
13	4-*n*-Amoxybenzoic Acid	60	1.14	+13	−2
15	4-Methoxy-2-hydroxybenzoic Acid	122	0.96	0	−11
16	4-Ethoxy-2-hydroxybenzoic Acid	151	1.10	−26	−10
17	4-*n*-Propoxy-2-hydroxybenzoic Acid	134	1.02	−5	−3
18	4-Isopropoxy-2-hydroxybenzoic Acid	127	1.10	−19	−2
19	4-*n*-Butoxy-2-hydroxybenzoic Acid	64	1.01	−52	−10
22	2,4-Diacetoxybenzoic Acid	134	1.01	−33	−3
24	4-Ethoxy-5-chloro-2-hydroxybenzoic Acid	103	1.07	−65	0
32	β-Methylumbelliferone	150	1.04	−43	−3
33	2,2′-Dihydroxybenzophenone	141	1.22	−1	+2
35	2-Hydroxy-3,5-dichlorobenzophenone	153	1.25	−59	−2
36	*p*-Aminosalicylic Acid	175	1.60	+111	−11
37	Streptomycin[b]	20 (hypo)	3.90	+123	+8.5

[a] Pathological ratio is the quotient of the average numerical pathological rating of the control animals divided by that of the treated animals.
[b] 40 mg. of streptomycin was given by hypo every two days.

derivatives has as yet been published. If it is borne in mind that, in animals, a portion of administered salicylate is converted to gentisic acid (15) and that gentisic acid has recently been used successfully for symptomatic treatment of rheumatic fever (16) some tentative hypotheses can be formulated. Gentisic acid is capable of entering into rapid-reversible oxidation-reduction reactions. In its oxidized (quinone) form it is also known to inactivate hyaluronidase *in vitro* (17). It is quite conceivable, then, that gentisic acid could accelerate certain oxidative metabolism and interfere with certain growth processes in tubercle bacilli. Similarly, some oxidized form of PAS such as 4-amino-2,5-dihydroxybenzoic acid or a further oxidation product could be, through its high and specific combining power, responsible for its bacteriostatic action. It is well known that many compounds which are capable of entering into para-quinoid oxidation-reduction systems are powerful and frequently very selective inhibitors of virulent tubercle bacilli. Examples include the para-alkoxy-benzenes and heterocycles studied by Feinstone, Friedman, and co-workers (18) and the 6-amino-2-substituted benzothiazoles studied by the present authors (19). It would seem then that virulent tubercle bacilli contain a system or systems which can interact, to the detriment of the organism, with many compounds which can form paraquinoid oxidation-reduction systems. The character of the additional substituents and their positionality is critically important. One fundamental approach to the chemotherapy of tuberculosis lies in the further synthesis and trial of a great variety of compounds which can give rise to para-oxidation-reduction systems and which bear additional substituents in various type patterns. The major problems are maintaining and heightening activity and selectivity while attempting to develop drugs of better *in vivo* absorption-distribution-excretion characteristics.

The lack of *in vivo* activity among the compounds reported here is undoubtedly due to unfavorable absorption-distribution-excretion factors. The carboxyl group in a great number of aromatic acids is readily conjugated and several instances are known in which aromatic ethers are readily dealkylated and conjugated *in vivo* (15). Further studies are in progress to extend this group of drugs and to attempt to find agents with better *in vivo* characteristics.

SUMMARY

1. Bacteriostatic values against *M. tuberculosis* H37Rv were determined for five simple phenols, twenty-six derivatives of benzoic and salicylic acids, two coumarins, and three benzophenones. Selected agents were tested against *M. tuberculosis* No. 607, *M. leprae*, *M. phlei*, a monilia, and *Trichophyton gypseum*.

2. Thirteen selected compounds were tested *in vivo* in guinea pigs infected with *M. tuberculosis* H37Rv using streptomycin and *p*-aminosalicylic acid as reference drugs.

3. Structure-bacteriostatic activity relations are analyzed and a theory of the mechanism of action of salicylic acid and some of its derivatives on tubercle bacilli is proposed.

4. Only two low *in vivo* activities were found. Further work is in progress.

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The Antiadrenolytic Action of Ergotamine*

By THEODORE O. KING† and THEODORE KOPPANYI

The vasomotor reversal of Dale is generally considered to be due to depression of the vasoconstrictor, but not of the vasodilator sympathetic neuro-effectors, leaving epinephrine free to stimulate the vasodilator, but not the vasoconstrictor sympathetic terminations. This phenomenon, called adrenolysis, may be caused by ergotamine, dibenamine, and other drugs. In dogs under nembutal anesthesia it was shown that ergotamine at certain dose levels not only does not reverse, but actually potentiates, the pressor effect of epinephrine. Moreover, when dibenamine and other powerful "sympatholytic" agents were used to produce vasomotor reversal, ergotamine in doses of 0.05 mg./Kg. or more restored the original pressor effect of epinephrine. Thus, ergotamine under certain experimental conditions reverses the effect of other adrenolytic drugs

In dogs, under pentobarbital anesthesia, ergotamine tartrate, in doses from 0.05 to 1.0 mg./Kg. or more, potentiates the pressor effects of epinephrine and also other sympathetic stimulants (2, 6). King (3) reported that ergotamine in similar doses converts the vasodepression produced by various doses of N-isopropyl-nor-epinephrine and N-isopropyl-nor-sympatol to marked pressor effects. These observations suggested the study of the effects of ergotamine on the vasomotor reversal of epinephrine induced by potent adrenolytic or sympatholytic agents in dogs anesthetized by sodium pentobarbital.

EXPERIMENTAL

Eleven animals were used for these experiments. At first the average hemodynamic responses were established by the intravenous injection of fixed amounts of epinephrine hydrochloride, nor-epinephrine hydrochloride, and of N-isopropyl-nor-epinephrine hydrochloride. Then the adrenolytic drug was injected either in a single dose or in divided doses by vein. In every case sufficient amounts were given to produce marked vasomotor reversal of epinephrine (criterion: the larger the dose of epinephrine, the greater the fall in blood pressure), to reduce or abolish the pressor effect of nor-epinephrine, and to increase the depressor effect of N-isopropyl-nor-epinephrine. Following the establishment of satisfactory vasomotor reversals, ergotamine tartrate was injected in doses of 0.05 mg./Kg., and in some cases this dose was increased to 0.2 mg./Kg. Five minutes after the administration of ergotamine, the injections of epinephrine, nor-epinephrine, and N-isopropyl-nor-epinephrine were repeated with the result that the epinephrine reversal was abolished and converted into a rise usually greater than the effect of the control injection. The nor-epinephrine pressor effects were also increased and the vasodepressor effects of N-isopropyl-nor-epinephrine were converted into marked pressor effects (Fig. 1). These pressor effects were not only sharp but of relatively long duration. Table I summarizes the effects of ergotamine on the vasomotor reversal and other effects produced by benzyl-imidazoline (Priscol), benzyl-β-chloroethylamine (Dibenamine), diethylaminomethylbenzodioxane (883 F), piperidinomethylbenzodioxane (933 F), and yohimbine hydrochloride.

The experiments were devised so as to exclude possible sources of error. In many cases the vasomotor reversing agent was administered and its effect tested. An hour later when vasomotor reversal was still present the full vasomotor reversing dose was again administered and only then was the effect of ergotamine studied on the responses to epinephrine, nor-epinephrine, and N-isopropyl-nor-epinephrine. This eliminated the possibility that ergotamine was counteracting the effect of a drug that is in part already essentially eliminated. The possibility that ergotamine produces vasospasm and thus generally

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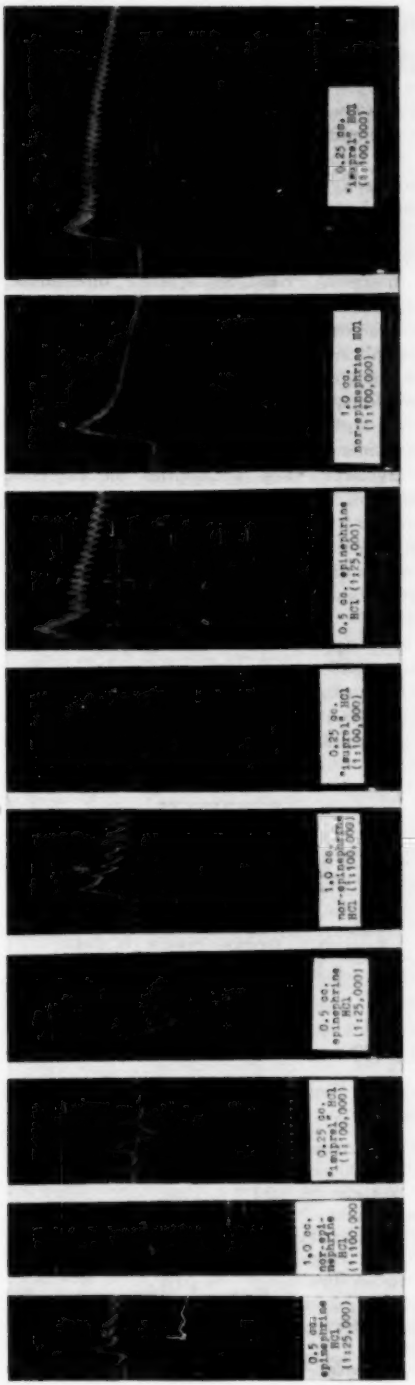
0.5 cc. epinephrine HCl (1:25,000)
1.0 cc. nor-epinephrine HCl (1:100,000)
0.25 cc. "isuprel" HCl (1:100,000)
0.5 cc. epinephrine HCl (1:25,000)
1.0 cc. nor-epinephrine HCl (1:100,000)
0.25 cc. "isuprel" HCl (1:100,000)
0.5 cc. epinephrine HCl (1:25,000)
1.0 cc. nor-epinephrine HCl (1:100,000)
0.25 cc. "isuprel" HCl (1:100,000)

Fig. 1.—Dog, 16.6 Kg., 35 mg. of sodium pentobarbital per kilogram by vein. Upper line represents mean arterial blood pressure recorded from the common carotid artery; middle line (base line, representing zero mm. of Hg pressure)—time, 5 seconds; lower line indicates injection of drugs.
1, 0.5 cc. epinephrine hydrochloride (1:25,000), vein. *2*, 1.0 cc. nor-epinephrine hydrochloride (1:100,000), vein. *3*, 0.25 cc. "isuprel" hydrochloride (1:100,000), vein; 6.0 mg. dibenamine hydrochloride per kilogram, vein, between *3* and *4*. *4*, 30 minutes later, same as in *1*. *5*, same as in *2*. *6*, same as in *3*; 0.05 mg. ergotomine tartrate per kilogram, vein, between *6* and *7*. *7*, 30 minutes later, same as in *1*. *8*, same as in *2*. *9*, same as in *3*.

abolishes vasodilator or vasodepressor effects was investigated. It was, indeed, shown that the depressor effects of acetylcholine and histamine were appreciably reduced by ergotamine administration (cf. 7). On the other hand, when vasospasm was produced by barium chloride (1.0 mg./Kg.) (cf. 5) or by Pitressin (cf. 4), the extent of vasodepression of epinephrine and N-isopropyl-nor-epinephrine was somewhat reduced but never completely abolished or reversed. Finally, when sodium nitrite (10 mg./Kg.) was administered intravenously the ergotamine effect on the vasomotor reversal and on N-isopropyl-nor-epinephrine vasodepression was preserved in spite of the profound general vasodilation. The duration of action of ergotamine in abolishing the vasomotor reversal is very prolonged and lasts for several hours. By this time the vasospasm, at least judged by the return of blood pressure to normal or subnormal levels, appeared to have diminished or disappeared.

DISCUSSION

Ahlquist, *et al.* (1), reported an antiadrenolytic action of ephedrine which caused the pressor effect of epinephrine to reappear after it had been blocked by Priscol. Their tracing indicates that Priscol did not reverse the pressor effect of 5γ of epinephrine but merely abolished it, and that following the administration of 5 mg. ephedrine, the same dose of epinephrine produced a rise smaller than the original. The most plausible explanations they offer for these phenomena are the resensitization of the pressor receptors by ephedrine or the blocking of the epinephrine depressor mechanism. Shaw and MacCallum (8) report that in cats, curare possesses the power of reversing the vasomotor reversing action of yohimbine. They offer the explanation that curare antagonizes yohimbine or that curare potentiates only the excitor or constrictor action of epinephrine and may antagonize the depressor or dilator action.

Apart from these isolated observations, the experiments reported above offer the first systematic proof that the vasomotor reversal caused by any type of adrenolytic or sympatholytic agent can be instantly reversed by another drug and, curiously, by a drug known to possess, under certain conditions, adrenolytic properties (2). The expected result should be an additive synergism and not an antagonism. Moreover, one can hardly escape the conclusion that unless there is a chemical antagonism between ergotamine and the other adrenolytic agents—and this assumption is hardly warranted—the adrenolytic and sympatholytic actions of dibenamine and other compounds, at least in the concentrations employed, are apparent rather than real. Furthermore, the experiments

TABLE I.—AVERAGE HEMODYNAMIC RESPONSES TO EPINEPHRINE, NOR-EPINEPHRINE, AND ISOPROPYL-NOR-EPINEPHRINE FOLLOWING THE ADMINISTRATION OF ADRENOLYTIC AGENTS AND ERGOTAMINE

	Blood-Pressure Changes in Mm. of Hg[a]		
	Control	After Adrenolytic	After Ergotamine (0.05–2.0 Mg./Kg.)
883 F, 6.0 Mg./Kg. (3 dogs)			
Epinephrine[b]	18	−18[a]	30
Nor-epinephrine[b]	26	12	44
N-Isopropyl-nor-epinephrine[b]	−15	−36	32
933 F, 12.0 Mg./Kg. (2 dogs)			
Epinephrine	17	−12	36
Nor-epinephrine	28	12	44
N-Isopropyl-nor-epinephrine	−32	−45	36
Dibenamine HCl, 6.0–10.0 Mg./Kg. (2 dogs)			
Epinephrine	15	−22	56
Nor-epinephrine	26	13	44
N-Isopropyl-nor-epinephrine	−14	−32	46
Priscol HCl, 7.0 Mg./Kg. (2 dogs)			
Epinephrine	27	−9	35
Nor-epinephrine	30	8	40
N-Isopropyl-nor-epinephrine	−23	−20	56
Yohimbine HCl, 7.0 Mg./Kg. (2 dogs)			
Epinephrine	24	−10	22
Nor-epinephrine	18	6	46
N-Isopropyl-nor-epinephrine	3, −12	−18	34

[a] Figures marked "−" indicate vasodepression.
[b] All doses of epinephrine, nor-epinephrine, and isopropyl-nor-epinephrine, 0.2 γ/Kg.

strongly suggest that the so-called adrenolytic drugs do not paralyze the excitor or constrictor sympathetic vasomotor receptors, but rather sensitize the depressor or dilator sympathetic or other receptors. This is particularly shown by the parallelism of the effects of the five adrenolytic agents and ergotamine on the effects of epinephrine and N-isopropyl-nor-epinephrine. In both cases it appears that the vasodepressor mechanism is sensitized by the five sympatholytic agents and desensitized by ergotamine. It is admitted that a part of this desensitization, at least during its initial phase, is due to the general vasospasm produced by ergotamine.

SUMMARY

The vasomotor reversal of epinephrine caused by Priscol, 883 F, 933 F, dibenamine, and yohimbine is abolished and the pressor effect restored by the administration of ergotamine.

Nor-epinephrine pressor effects are not reversed but merely greatly reduced or abolished by the adrenolytic agents. Ergotamine not only restores but exaggerates the pressor effects over the normal values.

In general, Priscol, 883 F, 933 F, dibenamine, and yohimbine, in adrenolytic doses, accentuate the vasodepression caused by N isopropyl-nor-epinephrine. Ergotamine not only abolishes these depressor effects but converts them to pressor effects.

The antiadrenolytic action of effective doses of ergotamine is of several hours' duration.

Ergotamine also antagonizes the depressor effects of acetylcholine and histamine. Drugs producing vasospasm, such as barium or Pitressin, do not possess antiadrenolytic action.

The antiadrenolytic action of ergotamine persists even when the vasospasm is counteracted by nitrites.

It is suggested that the adrenolytic effects of Priscol, 883 F, 933 F, dibenamine, and yohimbine, in doses used in these experiments, are not due to paralysis of motor sympathetic endings, but to a sensitization of the vasodepressor mechanism.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Indican
Coriamyrtin
L-Lyxose
9-Methyl-2,6,7-trihydroxy-3-fluorone
9-Phenyl-2,6,7-trihydroxy-3-fluorone
Phenyl pantothenone
4′-Methyl-3,4-benzepyrene
20-*tert*-Burylcholanthrene
15,20-Dimethylcholanthrene
20-Ethylcholanthrene
5-Nitro-3,4-benzpyrene
3-Chloro-20-methylcholanthrene
6-Cyano-20-methylcholanthrene
D-5-Ketogluconic acid
Hemocyanin
3,7,2′,4′-Tetrahydroxyflavone
3,7,8,2′-Tetrahydroxyflavone
1-Nitroacridine
1-Hydroxy-2-anthramine
1,6-Dihydroxynaphthalene
Sinigrin
Hemopyrrole

The Antipyretic Action of Some Derivatives of Salicylic Acid, *p*-Aminophenol, and Dihydropyrazolon*

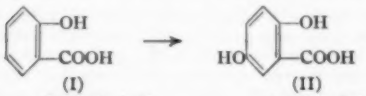
By C. I. JAROWSKI† and M. K. McPHAIL‡,§

The antipyretic actions in rabbits of six salicylate derivatives, an addition product of carbomethoxybenzoquinone and methyl gentisate have been determined and are reported. None of the compounds studied was significantly effective in lowering the temperature of the animals.

IT IS NOT KNOWN at the present time whether the action of drugs used as analgesics and antipyretics in rheumatic fever therapy results from the compounds *per se* or from some metabolites derived from their degradation. In the present study a number of compounds was prepared and evaluated for their ability to lower the temperature of rabbits made febrile by injection of a suspension of *Proteus vulgaris*.

The various compounds studied can be considered as belonging to one, and in some cases to two, of the following classes into which the analgesic-antipyretic drugs are usually divided (1): (I) the salicylate-cinchophen group; (II) the *p*-aminophenol derivatives; (III) the pyrazolon derivatives. The compounds belonging to the first two classes will be discussed under a single heading because of their structural relationship.

The Salicylate-p-Aminophenol Group.—The desirability of testing a metabolite of salicylic acid was indicated by a number of observations. Kapp and Coburn (2) showed that salicyluric, gentisic, salicylglucoronic, and salicylic acids were present in the urine of patients given sodium salicylate. Patients with acute rheumatic fever excrete less salicyl than normal subjects, but the gentisic acid concentration of the former group is two to four times greater than that found in healthy subjects. This finding suggests that the first step in the oxidative degradation of salicylic acid (I) is its conversion to gentisic acid (II).



(I) Salicylic Acid — (II) Gentisic Acid

The decreased excretion of salicyl in the rheumatic patient suggests that further degradation of gentisic acid occurs. Thus one might infer that the active therapeutic agent in alleviating rheumatic symptoms is either gentisic acid or one or more of its degradation products, a view which receives support from the recent findings of Meyer and Ragan (3) that sodium gentisate possesses antirheumatic activity equal to, or greater than, that of salicylate. Indirect evidence for this assumption is also suggested by the fact that the methyl ether of salicylic acid (III) (4) and 5-methyl salicylic acid (IV) (5) are less effective antirheumatic drugs, and neither compound could easily be converted to gentisic acid under biological conditions.

(III) Methyl Ether of Salicylic Acid — (IV) 5-Methyl Salicylic Acid

Likewise neither meta- nor para-hydroxy benzoic acids are effective antirheumatic agents (5). It is to be noted that the carboxyl group in the former compound would inhibit its conversion to gentisic acid since its meta-orienting influence would tend to inactivate the position para to the phenolic group. Thus there is some indirect evidence which appears to indicate that salicylate compounds which cannot easily be converted to gentisic acid do not possess good antirheumatic properties.

In addition to studying the action of gentisic acid for the reasons mentioned above, 5-aminosalicylic acid, 5-amino ethyl salicylate hydrobromide, and 5-amino methyl salicylate hydrobromide were included because it was of interest to determine the activity of compounds which could be considered as *p*-aminophenol as well as salicylic acid type compounds. These substances could also be degraded in the same manner as gentisic acid under biological conditions.

Since it was found that mild oxidation of methyl gentisate converts it to carbomethoxybenzoquinone (V), which in the presence of excess methyl gentisate forms an addition compound (VI), it was also believed of interest to test the addition compound. (Carbomethoxy-benzoquinone (V) was not isolated and hence could not be tested.)

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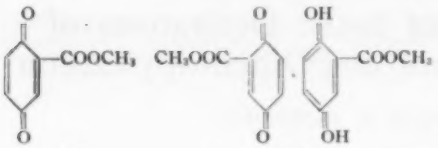
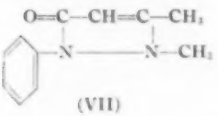
§ The authors are indebted to Mr. T. Wombolt for his technical assistance.



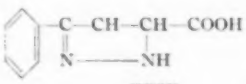
(V) (VI)

In addition to the above compounds salicyluric acid, a normal excretory product of patients receiving salicylate, was tested. Methyl gentisate was tested because of the possibility of its being less rapidly excreted and as a consequence more effective than gentisic acid.

The Pyrazolon Derivatives.—Since antipyrine (VII) is also an analgesic-antipyretic derivative, a dihydropyrazolon derivative (VIII) was prepared to determine its activity. It will be noted that in this derivative the phenyl group is attached to carbon in the heterocyclic ring while in antipyrine it is attached to nitrogen.



(VII)



(VIII)

Aspirin and amidopyrine were included in the tests, since aspirin is a representative of the salicylate-cinchophen group and amidopyrine is an example of the *p*-aminophenol derivatives.

EXPERIMENTAL

Preparation of Compounds.—The following compounds were prepared by methods described in the literature: gentisic acid (6), addition product of carbomethoxybenzoquinone and methyl gentisate (7), 2-carboxy-5-phenyl-dihydropyrazolon (8), salicyluric acid (9), and 5-amino salicylic acid (10).

Preparation of Pyrogen.—*Proteus vulgaris* was grown on nutrient agar for twenty-four hours at 37°. The growth was washed off in physiological saline and the concentration of organisms standardized to contain 10 billion per cc. This stock suspension was preserved with 0.5% phenol and stored at 4°. For the actual test, the stock suspension was diluted with sterile saline to contain approximately 2×10^7 organisms per cc., and one cc. of this suspension was injected into the marginal ear vein of rabbits to produce pyresis. This dose caused a 1° to 2° rise in temperature which lasted two to four hours.

Test Procedure.—All drugs were given orally suspended in 10% gum acacia, except where otherwise noted. In most cases 3 animals were given the drug and 3 were given the acacia suspension as controls for each test. In conducting the tests the rabbits were immobilized in stocks (11) and their rectal temperatures recorded (with a Centigrade thermometer graduated in 0.1° units) for 2 successive periods, thirty minutes apart. Then the pyrogen and drug were administered and the rectal temperatures recorded every half hour for the following five or six hours while the animals remained in the stocks. Since Beeson (12) has reported that rabbits injected daily with pyrogenic material show a progressive diminution in febrile response, it was believed important to avoid such a possibility as this in our work and therefore the animals were given rest periods of several days to a week between tests.

RESULTS

Statistical Treatment.—The data were treated in the following manner: The temperature departures (plus or minus) from the initial or zero temperature, averaged over the test period of five to six hours, were tabulated for all animals of the experimental group and compared with the same figures for the control group. Since the only distinction between the two was that the first received the test drug and the second an inert suspension, then the difference between them should express the antipyretic potency of the test compound. The analysis was made by using "Student's" t test which examines the probability that the differences found between experimental and control groups can be accounted for on the basis of random sampling alone. The average temperature departures from the normal for the two groups for all compounds tested are given in Table I, along with the t values, the probability p, and the significance. Probability values of 0.05 or less were considered to be significant.

Graphs.—In addition to the statistical analysis, graphs were prepared for each drug tested showing the average initial temperature, considered as zero, and the average rise or fall (plus or minus) from the initial temperature at thirty-minute intervals during the succeeding five or six hours for both the experimental and control groups of animals. Examples of the types of graph prepared are shown in Figs. 1 and 2. Figure 1 is shown to demonstrate the degree of pyrexia produced, and the antipyretic action of amidopyrine. Figure 2 represents the negative type of response found with all of the compounds tested, with the exception of amidopyrine.

DISCUSSION AND CONCLUSIONS

It is evident from an examination of Table I that the only drug studied which showed a statistically significant antipyretic action was amidopyrine when administered in a dose of 200 mg./Kg. (Experiments 9 and 10). When this compound was given in a dose of 100 mg./Kg. (Experiment 15) the result was not statistically significant although antipyresis occurred as was evident from the graph (not shown) and the average temperature changes of the experimental and control groups (columns 6 and 7). Acetylsalicylic acid did not have a statistically significant antipyretic action although in the two experiments in which it was given the treated animals had slightly lower temperatures than their controls. 5-Amino methyl salicylate

TABLE I.—ANTIPYRETIC ACTIVITY OF SOME DERIVATIVES OF SALICYLIC ACID, *p*-AMINOPHENOL, AND DIHYDROPYRAZOLON

Expt. No.	Compound	Dose Mg./Kg.	No. Experimental Animals	No. Control Animals	Average Temperature Departures from Normal (°C). Experimental Animals	Control Animals	*t* Value	*p* Value	Significance[a]
1	Gentisic Acid	200	1	3	+0.57[c]	+1.01	1.20	0.3	N. S.
2	Gentisic Acid (Na Salt)[b]	100	1	..					
3	Gentisic Acid (Na Salt)[b]	222.5	1	3					
4	5-Aminosalicylic Acid	250	4	2	+0.67	+0.66	0.06	0.9	N. S.
5	5-Amino Ethyl Salicylate HBr	500	4	2	+0.57	+0.65	0.54	0.6	N. S.
6	5-Aminosalicylic Acid	250	3	3	+1.18	+1.08	0.27	0.8	N. S.
7	Acetylsalicylic Acid	250	3	3	+1.13	+1.32	0.73	0.5	N. S.
8	Acetylsalicylic Acid	250	3	3	+1.09	+1.28	0.65	0.6	N. S.
9	Amidopyrine	200	3	3	−0.24	+0.98	3.56	0.02	S.
10	Amidopyrine	200	3	3	+0.12	+1.38	4.70	0.01	S.
11	5-Amino Methyl Salicylate HBr	250	3	3	+0.43	+0.24	0.39	0.7	N. S.
12	Methyl Gentisate	250	2	3	+1.05	+0.78	1.48	0.25	N. S.
13	Dihydropyrazolon Der. (VIII)	200	3	3	+1.24	+1.33	0.39	0.7	N. S
14	Addition Product (VI)	100	2	2	+0.42	+0.33	0.32	0.8	N. S.
15	Amidopyrine	100	2	2	+0.06	+0.33	1.59	0.25	N. S
16	Salicyluric Acid	250	3	3	+0.66	+0.63	0.07	0.9	N. S.

[a] N.S. = not significant; S. = significant.
[b] Sodium salt given intravenously in saline.
[c] Treated statistically as a single experiment.

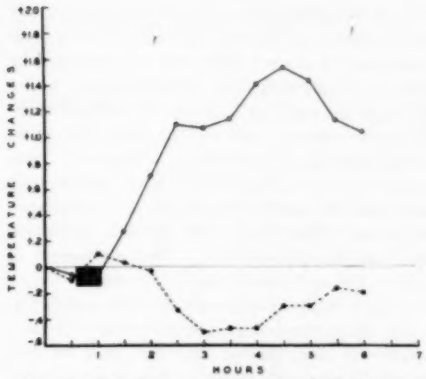


Fig. 1.—The lower curve (broken line) illustrates the average temperature changes of 3 rabbits given 200 mg./Kg. of amidopyrine in 10% gum acacia; the upper curve (solid line), the temperature changes of 3 control rabbits giving acacia only. The hatched area indicates the period of drug administration. Amidopyrine caused a fall in temperature well below the initial temperature; the pyrogen, an increase in temperature of about 1.5°.

hydrobromide caused a slight elevation in temperature and methyl gentisate a somewhat greater rise. Neither of these differences, however, proved to be statistically significant and the drugs were not investigated further. Rabbits given gentisic acid had lower temperatures than the controls but the difference between the two groups was not found to be significant. Unfortunately it was possible to administer gentisic acid only to a single rabbit on each of three experiments and the test probably suffers as a result.

The drugs which we have studied belong to either the salicylate-cinchophen, *p*-aminophenol, or pyrazolon group, members of which generally are considered to possess both analgesic and antipyretic activity. As none of the compounds tested showed marked antipyretic powers, it was assumed, when the work was first undertaken, that they would possess no important analgesic action and would, therefore, be of little value in the treatment of rheumatic fever. In view of Meyer and Ragan's recent report (3), however, on the antirheumatic action of sodium gentisate it would appear that this assumption is not tenable and that the antipyretic action

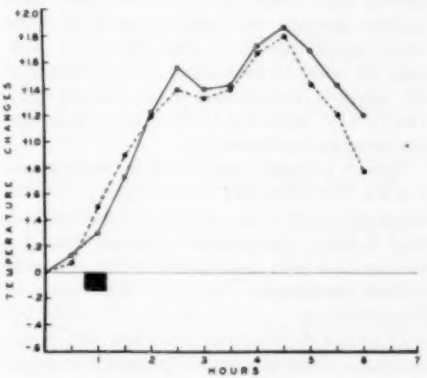


Fig. 2.—The broken line illustrates the average temperature changes of 3 rabbits given 200 mg./Kg. of the dihydropyrazolon derivative VIII in 10% gum acacia; the solid line, the temperature changes of 3 rabbits given acacia only. The hatched area indicates the period of drug administration. The ineffectiveness of compound VIII is apparent.

of a compound cannot be used as a criterion for its activity in the rheumatic state. In other words, the change of pain threshold cannot be measured by the change of heat threshold.

SUMMARY

Six salicylate derivatives, 3 pyrazoline compounds, and an addition product of carbomethozy-benzoquinone and methyl gentisate were tested for their antipyretic action in rabbits. None of these compounds was effective in lowering the temperature of the animals to a significant degree.

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The Characterization of the Trialkylbarbiturates*

By ALFRED HALPERN† and JAMES W. JONES‡

Based on experimental data involving the preparation of trialkylbarbituric acid derivatives and studies of degradative products of these compounds, a dynamic ionic structure for metallic derivatives of the barbiturates is proposed.

THE DYNAMIC PROPERTY of tautomerism has been consistently applied to the behavior of barbituric acid and its derivatives, yet the structure commonly used to designate the metallic derivatives is the static enol form. A review of the literature revealed many marked differences of opinion even to this static structure, with some authors favoring the keto-lactam form while others prefer the enol-lactim structure. Beilstein (1) refers to the sodium salt of barbital as the nitrogen derivative "or its desmotrope." The U. S. P. XIII and Merck's Index designate this structure as the enol.

There is a conspicuous absence in the literature of a 2,4- or 6-alkyl derivative resulting from the direct alkylation of the metal barbiturate with an alkyl halide. The products obtained from this reaction have been shown to be the 1,3- or 5- substituted compounds. Marotta and Rosanova (2) reported that they had obtained the enol ether as a result of the treatment of barbital with diazomethane. Their tautomeric product was subsequently shown to be identical with 1,3-dimethyl-5,5-diethylbarbituric acid prepared by Dox (3). The existence of the ether analogs has been demonstrated (4, 5) but these were not obtained by the direct alkylation of a barbiturate, rather through the reaction between the tetrachlorodihydropyrimidine and sodium alkoxide. The tetrachlorodihydropyrimidine, however, was prepared by treating barbital with phosphorous pentachloride and then converted to the tautomer of tetraethylbarbituric acid through alkylation with sodium ethoxide (5). This compound is quite stable and is not prone to rearrange. From analogy to related compounds, as isatin and carbostyril, the tautomeric enol form should be the less stable one, nevertheless, the ether, once formed, should be relatively stable, although intramolecular rearrangement may occur. The theoretical existence of the tautomers is established from the knowledge of bond energies which indicate that the C—O bond is more stable than the C—N bond (6).

Two isomeric methyl derivatives of isatin are known. The ether is obtained by converting potassium isatin into the silver salt and heating with methyl iodide. The N-analog may be obtained by the action of sodium hypobromite on N-methylindole. The constitution of these compounds was further established by the isolation of *o*-aminophenylglyoxylic acid from the hydrolysis of the ether and *o*-methylaminophenylglyoxylic acid from similar treatment of the N-substituted derivative. It was of interest to determine whether the tautomer of 1- or 3-alkylbarbital

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could be prepared through the analogous use of silver.

Since three tautomeric structures can be postulated for the monometallic derivatives of barbital, it is possible that the product which is usually described as the 1- or 3-alkylbarbital, resulting from the direct alkylation procedures, was in reality an equilibrium mixture of the N-substituted derivative and its O-analog. For the most part the reaction products isolated from the direct alkylation procedures were not investigated further to determine whether the ether analog might be present. An elementary analysis would not distinguish between the two tautomers.

This study was initiated to determine the possibility of the ether forming as a result of a direct alkylation procedure leading to the formation of a trialkylbarbituric acid derivative.

EXPERIMENTAL

Method A.—N-alkylbarbital was prepared through the reaction between ethyl diethylmalonate and the appropriate N-alkyl urea in the presence of sodium methoxide (7). The yields, melting points, and analysis of the various products are given in Table I.

Method C.—Molar equivalents of sodium barbital and an alkyl halide and 0.5 Gm. of copper powder were stirred for thirty minutes in 500 cc. of water at room temperature. The separated material was treated as in Method B (9).

This reaction is exothermic and has the advantage that there is no formation of a dialkylurea as was observed from the prolonged boiling of a barbituric acid with alkali (5).

Method D.—This was identical with Method C except that 1.236 Gm. of copper sulfate was substituted for the copper powder. The copper concentrations in both methods was equivalent.

Method E.—One-tenth mole of copper barbital was stirred with 0.2 mole of the alkyl halide for two hours. The mixture was extracted with ether. The extracts were dried over anhydrous sodium sulfate, the solvent removed, and the residue crystallized from diluted ethanol.

Method F.—One-tenth mole of silver barbital in 300 cc. of water was refluxed with 0.25 mole of alkyl halide for two hours and the mixture worked up as described in Method E.

Method G.—This process is identical with Method C except that 0.5 Gm. of moist silver hydroxide was substituted for the copper powder.

This procedure was also repeated under reflux and with 95% ethanol as the solvent.

Method H.—Sodium barbital was stirred with a 0.5 molar excess of the alkyl halide under reflux temperature for two hours. The mixture was cooled and

TABLE I.—THE PREPARATION OF TRIALKYLBARBITURIC ACID DERIVATIVES

	—% Theoretical Yield—					—Melting Point, °[a]—					—Nitrogen Analysis: Found %[b]—				
Method	Methyl	*i*-Propyl	*n*-Amyl	Benzyl	Allyl	Methyl	*i*-Propyl	*n*-Amyl	Benzyl	Allyl	Methyl	*i*-Propyl	*n*-Amyl	Benzyl	Allyl
A	76	74	67	71	62	155	117	36	127	74–75	14.2	12.5	11.1	10.2	12.0
B	48	46	40	44	42	155	117	36	127	74–75	14.0	12.3	10.9	10.16	12.23
C	75	71	70	73	72	155	117	35	127	74–75	13.9	12.1	10.8	...	...
D	50	56	48	46	44	155	117	36	127	74–75	...	12.0	11.0	10.35	12.3
E	13	18	10	16	12	154	117	36	127	74–75	...	12.2	11.1	10.10	...
E-reflux	23	30	24	28	26	154	117	36	127	74–75	...	...	11.0	...	...
E-ethanol	28	38	30	34	32	154	117	36	127	74–75	...	...	...	...	...
F	75	71	78	76	72	155	117	36	127	74–75	14.2	12.3	11.2	10.2	12.13
G	70	70	60	68	61	155	117	36	127	75	14.2	12.4	11.1	10.2	...
H	81	83	76	80	78	155	117	36	127	75	...	...	...	...	...
H + Cu	86	81	76	81	83	155	117	36	127	75	...	...	...	...	...
H + AgOH	83	88	82	86	88	155	117	36	127	75	14.1	12.54	11.3	10.15	...
J + Cu-barbital	84	80	76	79	81	155	117	36	127	74–75	13.8	12.1	...	...	...
J + Ag-barbital	93	84	80	85	86	155	117	36	127	74–75	...	12.2	11.1	10.11	...

[a] After three recrystallizations from diluted ethanol and one from benzene-petroleum ether mixture.
[b] The calculated nitrogen for these compounds are: methyl, 14.1; *i*-propyl, 12.3; *n*-amyl, 11.0; benzyl, 10.19; allyl, 12.0.

The direct alkylation of sodium barbital was then attempted by the following procedures. The alkyl halides used in these methods were methyl iodide (b. p. 41–42°), isopropyl bromide (b. p. 58–59°), *n*-amyl bromide (b. p. 127–129°), benzyl chloride (b. p. 71–73°/20 mm.) and allyl chloride (b. p. 45–46°). The melting points, yields, and analyses of the products resulting from the direct alkylation procedures are given in Table I.

Method B.—One-half mole of sodium barbital in 300 cc. of water was refluxed for four hours with 0.5 mole of the alkyl halide. The separated oily material hardened on standing in an ice chest and was filtered, dried, and recrystallized from diluted ethanol (8).

the sodium halide filtered off. The excess alkyl halide was distilled under reduced pressure and the residue crystallized from ethanol. The reaction was also carried out in the presence of copper powder and silver hydroxide.

Method J.—Copper barbital and silver barbital were substituted for sodium barbital of method H.

A mixed melting point of the products produced by the direct alkylation and those resulting from the condensation of ethyl diethyl malonate with the corresponding N-alkyl urea showed no depression of the melting point indicating that the products were identical. While this would seem to establish the structure of the direct alkylation product as a nitrogen derivative, the possibility still existed that these

compounds were equilibrium mixtures of the N-derivatives and the O-analog since urea has also been described as being tautomeric. This possibility, however, is extremely remote. Accordingly, the reaction mixture and its products were quantitatively analyzed.

Degradative analyses were carried out on the products resulting from methods A, B, C, E, F, G, and J. The following procedure was followed and alkylamine and diethylacetic acid were obtained in all instances, establishing the products as N-substituted.

Degradative Hydrolysis of the N-Alkylbarbital Derivatives.—One-tenth mole of the appropriate N-alkylbarbital was hydrolyzed at reflux temperature with ten equivalents of hydrochloric acid. The mixture was cooled and made alkaline with sodium hydroxide (Solution I) and steam distilled into an excess of 2*N* hydrochloric acid. The solution was evaporated to dryness and the residue dissolved in 150 cc. of 1*N* sodium hydroxide. The alkaline solution was fractionally distilled with a stream of air bubbling through the mixture into a known excess of 0.1*N* hydrochloric acid and the ammonia nitrogen determined (Table II).

The higher boiling fraction was steam distilled and the distillate treated with an excess of benzenesulfonylchloride in the presence of sodium hydroxide. The benzene sulfonamide was filtered, washed with water, and recrystallized from diluted ethanol. The physical constants of the benzene sulfonamides are recorded in Table II. A portion of the benzene sulfonamide was hydrolyzed with dilute sodium hydroxide and the amine isolated and its boiling point determined (Table II).

From the first alkaline solution above (Solution I) diethylacetic acid was isolated in 88–94% yields for the different compounds. No other products were found. This compound was identified through its anilid which melted at 193°. N: calculated 7.1, found 7.0; 6.9.

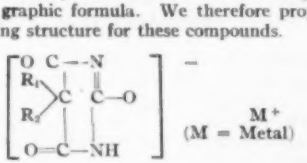
Since the absence of ammonia nitrogen would be conclusive proof of N-substitution, the 1,3-dialkyl-5,5-diethylbarbituric acid derivatives were prepared through methods A, C, G, and J, and treated in the manner described above, except that the period of refluxing was doubled. The result of the hydrolysis of the tetraalkyl derivatives are reported in Table II.

The results of the degradative procedures were of particular interest since only the N-substituted product was obtained through all the alkylation procedures. The failure to obtain the O-ether by the methods employing silver must be considered in any discussion of the tautomerism of these compounds. Similar treatment of isatin resulted in the formation of the tautomeric compounds. By comparison to

other imides having the —C—NH—C— group, as phthalimide and succinimide, it would appear that the dialkylbarbiturates behave in a like manner.

Dox (5) has stated that at least one of the lactam groupings has the properties of an imide since a monosodium salt can be prepared which reacts with the alkyl halide to produce an N-alkylated derivative. After alkylation of one lactam, the resulting 1,5,5-trisubstituted barbituric acid again behaves as an imide, with the remaining lactam forming a sodium salt which reacts as before. Thus 1,3,5,5-tetraalkyl derivatives can be prepared.

In view of the above results, it was felt that a dynamic structure would satisfy more of the properties of the metal dialkylbarbiturates than does the present static graphic formula. We therefore propose the following structure for these compounds.



This structure is in agreement with the observed behavior of the metal salts of barbituric acid derivatives and allows for the maximum interpretation of tautomerism as applied to these compounds.

SUMMARY

1. Trialkylbarbituric acid derivatives have been prepared through different methods.

2. From mixed melting points and degradative analytical data, the product of the direct alkylation of barbital was shown to be N-substituted. The tautomeric O-analog could not be isolated from the reaction products.

3. A dynamic ionic structure for the metallic derivatives of the barbiturates was proposed.

TABLE II.—NITROGENOUS PRODUCTS RESULTING FROM DEGRADATIVE ANALYSIS

Compound	Ammonia N %: Theoretical[a]	Ammonia N %: Found	Derived Amine	Yield, %	B. p.°	—N-alkylbenzenesulfonamide— M. p.°	N-Analysis[b]: Calcd., N, %	N-Analysis[b]: Found, N, %
Trialkylbarbituric acid Derivatives[c]								
1-Methyl	...		CH_3NH_2	...	...	29–30	8.2	7.9
1-Isopropyl	...		$(CH_3)_2CHNH_2$	93.4	33–35	25–26[d]	7.0	7.1
1-*n*-Amyl	96		$CH_3(CH_2)_4NH_2$	94.2	104	88–89	6.1	6.0
1-Benzyl	98.4		Benzylamine	97.3	185	87–88	5.6	5.7
1-Allyl	94		Allylamine	95.3	54–55	39	7.1	6.9
Tetraalkylbarbituric Acid Derivatives[e]								
1,3-Di-*n*-amyl	0		*n*-Amylamine	97.6	104	88–89	...	...
1,3-Dibenzyl	0		Benzylamine	99.1	185	87–88	...	...
1,3-Diallyl	0		Allylamine	98.2	54–55	39	...	...

[a] Based on the assumption that the unsubstituted amide is converted to ammonia during the acid hydrolysis. From the alkali fusion of barbital, ammonia and potassium cyanide were isolated (2).

[b] Average of two determinations with a deviation less than ±0.2.

[c] Refers to the 1-alkyl-5,5-diethylbarbituric acid derivatives.

[d] The phenyl thiourea derivative melted at 101° (Shriner, R. L., and Fuson, R. C., "Systematic Identification of Organic Compounds," J. Wiley & Sons, New York City, 1948, ed. 3, 179 pp.).

[e] Refers to the 1,3-dialkyl-5,5-diethylbarbituric acid derivatives.

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Notes

A Note on the Microscopic Characterization of the Polymorphic Forms of Ouabain*

By GEORGE L. KEENAN†,‡

IN 1929, Schwartze, Hann, and Keenan (1) published the results of an investigation of ouabain, principally for the purpose of recommending a form that could be utilized as a reliable U. S. P. Reference Standard. At that time it was suggested that ouabain, crystallized from water and containing 20% moisture, be supplanted by the drug crystallized from alcohol, to which a little ether had been added, and which contained approximately 12.5% moisture. At the present time this form of ouabain is recognized as the U. S. P. Reference Standard (2).

More recently, the writer has been interested in calling attention to the polymorphic forms of ouabain, as a result of the microscopic study of the drug crystallized from water and alcohol, respectively. The results of this investigation would also serve as additional identity tests for the drug, in addition to specific rotation and bioassays. These dimorphs are readily converted one into the other, even on an object slide. The conditions necessary for their existence and their optical crystallographic properties are presented as follows.

TESTS

Quadrilateral Plate Form.—This form, crystallized from water at room temperature, has been found to be the ouabain of commerce. The habit of the crystals is quadrilateral plates, showing very weak double refraction in parallel polarized light (crossed nicols), apparently crystallizing in the tetragonal system. The significant refractive indices of this form are: $\omega = 1.525$ and $\epsilon = 1.523$. It will be observed that these values differ only by 0.002; therefore, when the material is mounted on an object slide in either of the significant immersion liquids, the borders of the plates are almost invisible in ordinary light. By crystallizing this form from a drop of 95% alcohol on an object slide at room temperature, the rod-shaped habit of the drug is obtained (Fig. 1).

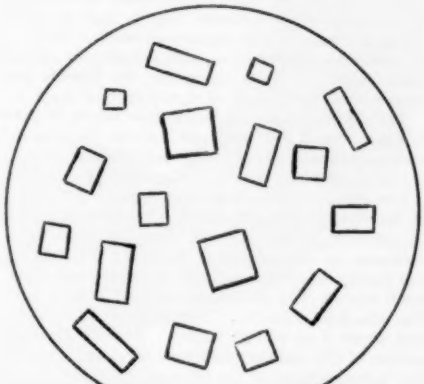

Fig. 1.—Typical habit from water.

Rod Form.—Elongated forms characterize the habit of the drug crystallized from 95% alcohol. These rods show strong double refraction, with parallel extinction and positive elongation (crossed nicols, supplemented by the selenite plate). The refractive indices are distinctly different from those

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‡ The writer acknowledges his indebtedness to the following for samples of ouabain: Committee of Revision of the Pharmacopœia of the United States of America 1940–1950; S. B. Penick & Company, New York, N. Y.; and Dr. K. K. Chen, The Lilly Research Laboratories, Indianapolis, Ind.; and to Baldwin-Wallace College, Berea, Ohio, for use of apparatus.

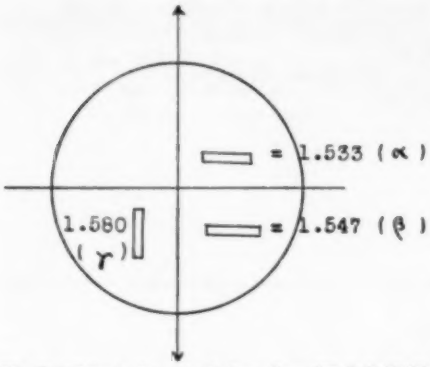

Fig. 2.—Orientation of rod-shape form (U. S. P. XIII Reference Standard).

characteristic of the quadrilateral form, and are as follows: $\alpha = 1.533$, $\beta = 1.547$, $\gamma = 1.580$ (probably orthorhombic); all ± 0.003 (Fig. 2).

This rod-shaped form is that characteristic of the U. S. P. Reference Standard, and, as already suggested, has optical crystallographic characters that are useful as microscopic identity tests.

SUMMARY

Attention has been called to the dimorphic forms of ouabain, the quadrilateral plates crystallized from water and the rods from alcohol. The optical crystallographic characters significant for rod-shaped form serve as additional identity data for the U. S. P. Reference Standard.

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A Modified Squibb Separatory Funnel for Microanalytical Work*

By CHARLES F. BICKFORD†

THE SQUIBB pear-shape type separatory funnel of about 30 cc. capacity, modified as shown in the accompanying diagram, Fig. 1, may be used advantageously in microanalytical work, when it is necessary to collect into a small volume, for critical observation and sharp separation, the usually less than a cubic centimeter of titrant solvent layer.

The modification comprises a short length of glass tubing about 2 cm. long and 0.5 cm. internal diameter, placed between the pear-shaped portion of the separatory funnel and the stopcock.

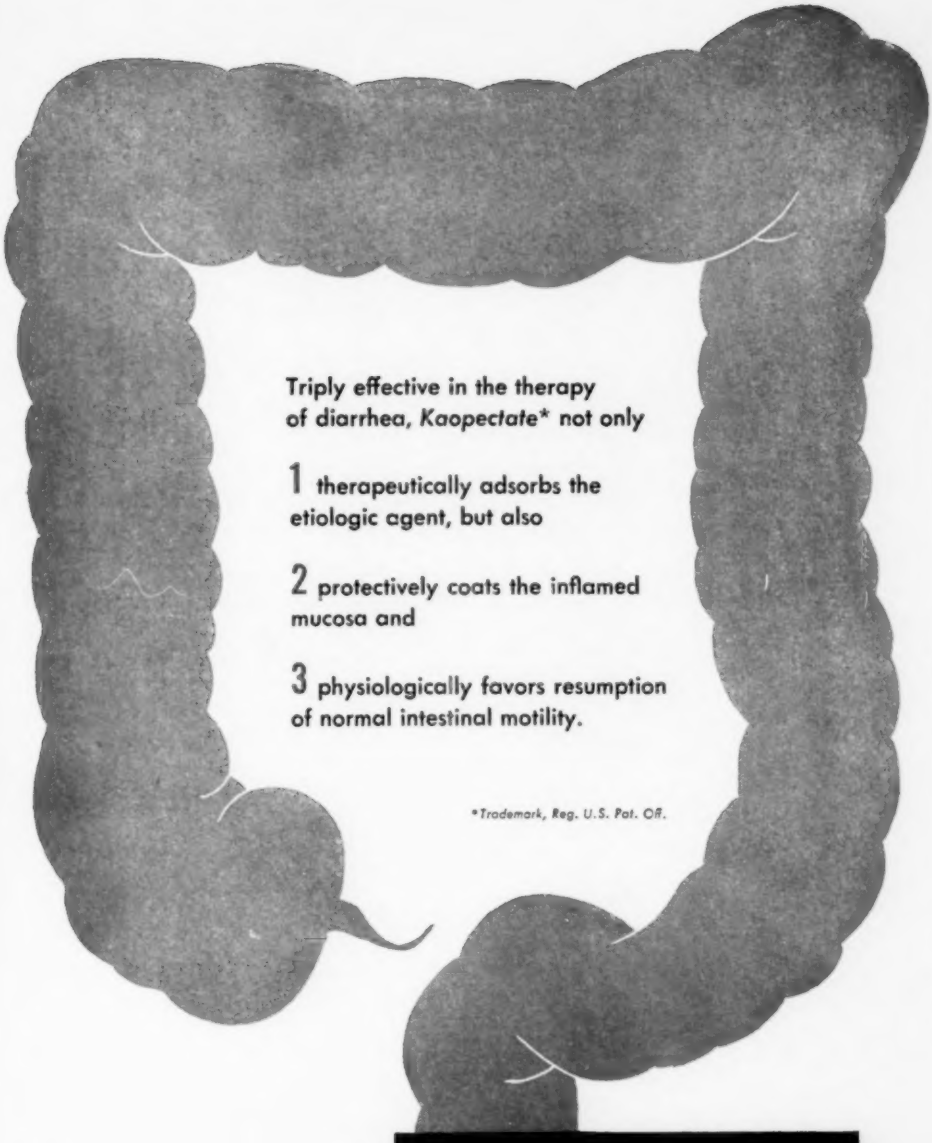
The value of the modification may be illustrated by describing a final step in the estimation of several micrograms of mercury. A standard solution of dithizone in chloroform is employed to estimate and extract simultaneously the mercury present in a small volume of approximately normal sulfuric acid. The standard solution of dithizone is adjusted so that about 2 cc. will be needed. An initial 1.4 cc. portion of the standard dithizone solution is added, from a micro-buret, to the solution in the separatory funnel. The two layers are shaken until all the dithizone has reacted with the mercury. The chloroform layer is separated. This procedure is repeated with 0.2 cc. portions of the standard dithizone solution until the presence of unreacted dithizone changes the characteristic orange-yellow color of mercury dithizonate in chloroform. This end point is reproducible to 0.1 cc., sufficiently accurate for routine work. The chloroform layer, filling about 1 cm. length of the straight glass tube portion above the stopcock, may be easily observed. A definite color change may be ascertained rapidly.

Fig. 1.—Microanalytical separatory funnel.

The separatory funnel may be employed in a similar manner when dithizone is used to determine microgram quantities of other metals.

* Received November 10 from the Pharmaceutical Development Division, E. R. Squibb & Sons, Brooklyn, N. Y.

† Research Associate, Pharmaceutical Formulations Department.



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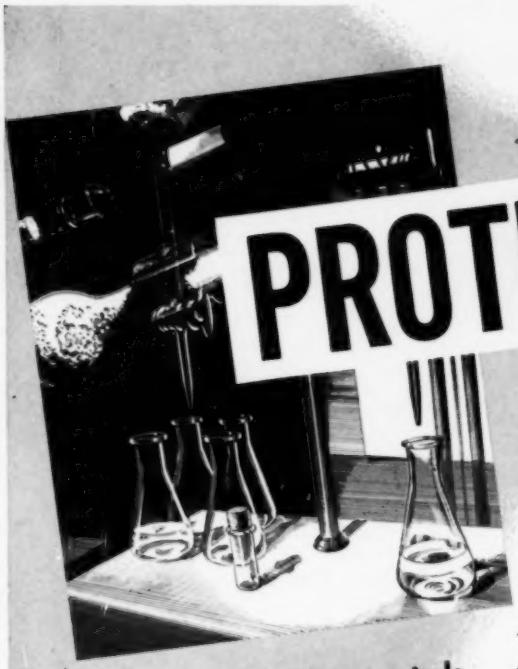

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